

# **PULMONARY FIBROSIS**

By Malvina Anna Krupiczojc

A thesis submitted to the University of London for the degree of  
of Philosophy

Centre for Respiratory Research  
Rayne Institute, University College London, 5 University Street, 1  
WC1E 6JJ (UK)

UMI Number: U591593

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U591593

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.  
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against  
unauthorized copying under Title 17, United States Code.



ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

---

## **ACKNOWLEDGEMENTS**

This thesis would have not been written without the help and support of a number of people. I would like to take this opportunity to thank several of them in particular.

First, my supervisor, Dr Rachel Chambers, whose incredible guidance, knowledge and support over the past three years has been endless and without whom the completion of this thesis would not have been possible. For nurturing my passion for science and for always being there as a mentor and providing invaluable insight in critically reviewing this thesis.

Dr Chris Scotton, my second supervisor, for teaching me everything I know, for providing invaluable guidance and support, for critically reviewing, proof reading and formatting this thesis and for friendship.

Professor Geoff Laurent, in whose laboratory this thesis was carried out, for his constant encouragement and enthusiasm.

Steve Bottoms, for all his teaching and advice on histological techniques.

All my colleagues in the Centre for Respiratory Diseases, with special thanks to Dr Paul Mercer and Xiaoling Deng for the great discussions, friendship and social activities.

My brother, Bartosch, for phoning me up with words of support from far off places.

All my friends, with special thanks to Beatrice and Freddy for their extraordinary patience and for helping with the preparation of this thesis.

My parents, for their unconditional support for as long as I can remember.

## **Declaration**

I confirm that the work contained in this thesis is entirely my own.

---

## ABSTRACT

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders, characterised by the excessive deposition of extracellular matrix proteins within the pulmonary interstitium. Current evidence suggests that the differentiation of fibroblasts into highly synthetic and contractile myofibroblasts plays a central role in the pathogenesis of pulmonary fibrosis. Uncontrolled coagulation activity with extravascular expression of tissue factor (TF) leading to the activation of coagulation zymogens, including FVII, FX and prothrombin, has been documented in the lungs of patients with pulmonary fibrosis. In addition to their role in blood clotting, coagulation proteinases induce multiple pro-inflammatory and pro-fibrotic cellular effects via the activation of their major signalling receptors the PARs, including PAR<sub>1</sub>. Until recently, coagulation zymogens were thought to be exclusively derived from the circulation and then locally activated in response to tissue injury. This thesis shows for the first time that FX is locally upregulated in human and murine fibrotic lung tissue and that the alveolar epithelium represents a prominent cellular source of this protein. This thesis further reports that FXa is a potent inducer of the myofibroblast differentiation programme via PAR<sub>1</sub> and the transcriptional upregulation of thrombospondin-1 leading to the activation of TGF- $\beta$ <sub>1</sub>. The work presented here further demonstrates that PAR<sub>1</sub>, TSP-1 and  $\alpha$ -SMA co-localize to fibrotic foci in idiopathic pulmonary fibrosis and a direct causal link between FXa and the development of fibrosis was demonstrated by showing that a direct FXa inhibitor attenuated bleomycin-induced lung fibrosis. This thesis therefore identifies a novel pathogenic mechanism by which FXa, a central proteinase of the coagulation cascade, is locally upregulated and drives the fibrotic response to lung injury. These findings herald a paradigm shift in the current understanding of the tissue origin of excessive procoagulant activity and further place PAR<sub>1</sub> central to the crosstalk between pro-coagulant signalling and tissue remodelling.



---

*To my parents and my brother*

---

## TABLE OF CONTENTS

TITLE PAGE.....	1
ABSTRACT.....	2
ACKNOWLEDGEMENTS.....	3
TABLE OF CONTENTS.....	5
LIST OF FIGURES.....	12
LIST OF TABLES.....	15
LIST OF ABBREVIATIONS.....	16

---

## CHAPTER 1: INTRODUCTION..... 19

---

1.1. Pulmonary Fibrosis.....	19
1.2. Idiopathic Pulmonary Fibrosis (IPF).....	21
1.3. Pathogenesis of Pulmonary Fibrosis.....	23
1.3.1. <i>The role of inflammation in the pathogenesis of IPF</i> .....	26
1.4. Extracellular matrix deposition in the fibrotic lung.....	29
1.4.1. <i>Collagen structure</i> .....	29
1.4.2. <i>Collagen deposition in a normal and fibrotic lung</i> .....	30
1.5. The myofibroblast and pulmonary fibrosis.....	31
1.5.1. <i>Discovery and origin of the myofibroblast</i> .....	31
1.5.2. <i>The role of myofibroblast in pulmonary fibrosis</i> .....	33
1.5.3. <i>Origin of myofibroblasts</i> .....	35
1.5.4. <i>Regulation of myofibroblast differentiation</i> .....	37
1.6. Role of TGF- $\beta$ in myofibroblast differentiation and pulmonary fibrosis	37
1.6.1. <i>TGF-<math>\beta</math> function and signalling</i> .....	37
1.6.2. <i>The latent TGF-<math>\beta_1</math> complex</i> .....	39
1.6.3. <i>Activation of TGF-<math>\beta_1</math> by TSP-1</i> .....	42
1.6.4. <i>TGF-<math>\beta_1</math> and fibrosis</i> .....	44
1.7. The role of the coagulation cascade in fibrosis.....	46
1.7.1. <i>The coagulation cascade</i> .....	46
1.7.2. <i>Regulation of the coagulation cascade</i> .....	49

---

1.7.3. Evidence for the role of the coagulation cascade in pulmonary fibrosis.....	50
1.7.4. Fibrinolysis in the fibrotic lung.....	51
1.7.5. Targeting the coagulation cascade.....	52
1.7.6. Mechanisms by which the coagulation cascade exerts its profibrotic effects.....	52
1.8. Coagulation Factor X.....	53
1.8.1. Structure.....	53
1.8.2. FX activation.....	56
1.8.3. Biological functions of FXa.....	57
1.9. Proteinase Activated Receptors (PARs).....	57
1.9.1. PAR <sub>1</sub> .....	59
1.9.2. PAR <sub>1</sub> signalling pathways.....	60
PAR <sub>1</sub> -Gα <sub>i</sub> Pathway.....	61
PAR <sub>1</sub> -Gα <sub>q</sub> Pathway.....	63
PAR <sub>1</sub> -Gα <sub>12/13</sub> Pathway.....	63
1.9.3. Regulation of PAR <sub>1</sub> signalling.....	64
1.10. Role of PAR <sub>1</sub> in fibrosis.....	65
1.10.1. PAR <sub>1</sub> and inflammation.....	65
1.10.2. PAR <sub>1</sub> exerts potent pro-fibrotic effects in vitro.....	66
1.10.3. PAR <sub>1</sub> exerts potent pro-fibrotic effect in vivo.....	69
1.11. Summary and hypothesis.....	70
 Chapter 2: Materials & Methods.....	 71

---

## Materials

2.1. Chemicals, solvents and tissue culture materials.....	71
2.2. Coagulation factors, cytokines, growth factors and peptides.....	71
2.3. Inhibitors and Antibodies.....	71
2.4. Alveolar and bronchial epithelial cells, fibroblasts and tMLEC.....	72

## Methods

2.5. Animals.....	73
-------------------	----

---

<b>2.6. Animal Model of Pulmonary Fibrosis.....</b>	<b>74</b>
<b>2.6.1. Oropharyngeal instillation of bleomycin.....</b>	<b>74</b>
<b>2.6.2. Aerosolized instillation of bleomycin.....</b>	<b>74</b>
<b>2.7. BALF FXa activity.....</b>	<b>75</b>
<b>2.8. FXa inhibition <i>in vivo</i> using ZK 807834.....</b>	<b>75</b>
<b>2.9. Determination of total lung collagen.....</b>	<b>76</b>
<b>2.9.1. Pre-column Derivatisation.....</b>	<b>76</b>
<b>2.9.2. Instrumentation and chromatography conditions.....</b>	<b>77</b>
<b>2.10. RT-PCR and real-time RT-PCR analysis.....</b>	<b>78</b>
<b>2.10.1. Precautions taken to prevent RNA degradation.....</b>	<b>78</b>
<b>2.10.2. RNA extraction.....</b>	<b>79</b>
<b>2.10.3. DNase treatment.....</b>	<b>79</b>
<b>2.10.4. cDNA synthesis.....</b>	<b>80</b>
<b>2.10.5. Primer design.....</b>	<b>80</b>
<b>2.10.6. RT-PCR.....</b>	<b>81</b>
<b>2.10.7. Real-time RT-PCR.....</b>	<b>82</b>
<b>2.11. Histological Analysis.....</b>	<b>83</b>
<b>2.11.1. Preparation of slides.....</b>	<b>83</b>
<b>2.11.2. Immunohistochemical localization of FX, TSP-1, PAR<sub>1</sub> and <math>\alpha</math>-SMA</b>	<b>83</b>
<b>2.11.3. Demonstration of lung architecture.....</b>	<b>83</b>
<b>2.12. Patient samples.....</b>	<b>84</b>
<b>2.12.1. Giessen cohort.....</b>	<b>84</b>
<b>2.12.2 Brompton cohort.....</b>	<b>84</b>
<b>2.12.3 Pittsburgh cohort.....</b>	<b>84</b>
<b>2.13. Laser-capture microdissection.....</b>	<b>85</b>
<b>2.14. Alveolar epithelial cell isolation.....</b>	<b>85</b>
<b>2.15. Fibroblast isolation.....</b>	<b>85</b>
<b>2.16. Cell culture conditions.....</b>	<b>86</b>
<b>2.17. Preparation of agonists and antagonists.....</b>	<b>86</b>
<b>2.18. Cell preparation for experiments.....</b>	<b>87</b>
<b>2.19. Western blot analysis of <math>\alpha</math>-SMA, pSMAD/SMAD, TSP-1 and ERK2...</b>	<b>87</b>
<b>2.19.1. Cell culture conditions and sample collection.....</b>	<b>87</b>

---

2.19.2. <i>BCA protein assay</i> .....	88
2.19.3. <i>Western blotting procedures</i> .....	88
2.19.4. <i>Protein Detection</i> .....	89
2.19.5. <i>Quantification of western blot</i> .....	89
2.20. <i>Immunocytochemical visualisation of actin fibres</i> .....	90
2.21. <i>Measurement of TGF-<math>\beta</math> activity with the tMLEC assay</i> .....	90
2.21.1. <i>Principles of assay</i> .....	90
2.21.2. <i>Co-cultures</i> .....	91
2.21.3. <i>Cell culture supernatants</i> .....	91
2.22. <i>Methylene blue proliferation study</i> .....	92
2.22.1 <i>Principles of the assay</i> .....	92
2.22.2. <i>Cell culture conditions</i> .....	92
2.22.3. <i>Methylene Blue assay procedure</i> .....	92
2.23. <i>Statistical analysis and expression of data</i> .....	93
<b>CHAPTER 3: RESULTS</b> .....	94

---

## Overview

3.1. <i>Local generation of coagulation zymogen FX in the lung</i> .....	94
3.1.1. <i>Introduction</i> .....	94
3.1.2. <i>FX gene expression in bleomycin-induced lung injury and fibrosis</i>	94
3.1.3. <i>FX gene expression in pulmonary fibrosis induced by aerosolised bleomycin</i> .....	95
3.1.4. <i>FX expression in isolated alveolar epithelial cells post bleomycin-induced lung injury and fibrosis</i> .....	98
3.1.5. <i>Immunohistochemical localization of FX/FXa protein in bleomycin-induced lung injury and fibrosis</i> .....	100
3.1.6. <i>BALF FXa levels in bleomycin-induced lung injury and fibrosis...</i>	102
3.1.7. <i>The zymogen FX is expressed in the human lung and is upregulated in IPF</i> .....	103
3.1.8. <i>Immunohistochemical localization of FX/FXa in IPF</i> .....	106
3.1.9. <i>FX expression in human alveolar and bronchial epithelial cells in vitro</i> .....	109
3.1.10. <i>Bleomycin induces FX expression in alveolar epithelial cells</i> .....	109

---

3.1.11. Summary.....	113
<b>3.2. The role of FXa in fibroblast to myofibroblast differentiation.....</b>	<b>114</b>
3.2.1. Introduction.....	114
3.2.2. Effect of FXa on primary adult human lung fibroblast to myofibroblast differentiation.....	114
3.2.3. Kinetics of myofibroblast differentiation induced by FXa.....	115
3.2.4. Effects of FXa on foetal human and mouse lung fibroblast to myofibroblast differentiation.....	118
3.2.5. FXa and Thrombin have similar stimulatory effects on myofibroblast differentiation.....	120
3.2.6. Effects of FXa on primary human adult lung fibroblast $\alpha$ -SMA mRNA levels.....	120
3.2.7. Effect of FXa on $\alpha$ -SMA fibre formation on human adult lung fibroblasts.....	123
3.2.8. Summary.....	127
<b>3.3. The role of PAR<sub>1</sub> in FXa-induced myofibroblast differentiation.....</b>	<b>128</b>
3.3.1. Introduction.....	128
3.3.2. FXa-induced myofibroblast differentiation is independent of thrombin activity.....	128
3.3.3. The effect of antistasin on FXa-induced myofibroblast differentiation.....	130
3.3.4. Confirmation of PAR <sub>1</sub> and PAR <sub>2</sub> expression at mRNA level in lung fibroblasts.....	130
3.3.5. PAR <sub>1</sub> but not PAR <sub>2</sub> activation is sufficient for fibroblast to myofibroblast differentiation.....	133
3.3.6. PAR <sub>1</sub> activation is necessary for FXa-induced myofibroblast differentiation.....	136
3.3.7. Effect of pertussis toxin (PTX) and the PAR <sub>1</sub> -G $\alpha_q$ inhibitor (q94) on FXa- induced $\alpha$ -SMA expression.....	138
3.3.8. Summary.....	142

---

<b>3.4. The role of TGF-<math>\beta</math> in FXa-induced myofibroblast differentiation.....</b>	<b>143</b>
3.4.1. <i>Introduction.....</i>	143
3.4.2. <i>FXa does not regulate TGF-<math>\beta</math> mRNA levels.....</i>	143
3.4.3. <i>TLLR-NH<sub>2</sub> induces activation of TGF-<math>\beta</math>.....</i>	144
3.4.4. <i>FXa induces Smad2/3 phosphorylation in pHALFs.....</i>	144
3.4.5. <i>FXa stimulates thrombospondin-1 (TSP-1) expression at the mRNA and protein level.....</i>	149
3.4.6. <i>PAR<sub>1</sub> mediates TGF-<math>\beta</math> activation in a TSP-1 dependent manner.....</i>	152
3.4.7. <i>FXa-induced <math>\alpha</math>-SMA expression is dependent on TGF-<math>\beta</math> activity.....</i>	153
3.4.8. <i>PAR<sub>1</sub>, <math>\alpha</math>-SMA and TSP-1 colocalise in human fibrotic lesions.....</i>	153
3.4.9. <i>Summary.....</i>	159

<b>3.5. Effect of the direct FXa inhibitor ZK 807834 on FXa-induced pro-fibrotic effects <i>in vitro</i> and <i>in vivo</i>.....</b>	<b>160</b>
3.5.1. <i>Introduction.....</i>	160
3.5.2. <i>Effect of ZK 807834 on myofibroblast differentiation by FXa.....</i>	160
3.5.3. <i>Effect of ZK 807834 on bleomycin-induced lung injury and fibrosis..</i>	163
3.5.4. <i>Effect of oropharyngeal instillation of ZK 807834 on bleomycin-induced lung injury and fibrosis.....</i>	166
3.5.5. <i>Summary.....</i>	169

---

## CHAPTER 4: DISCUSSION..... 170

---

### Overview

<b>4.1. Local FX generation in the lung.....</b>	<b>172</b>
4.1.1. <i>FX is expressed locally in the lung and is upregulated in fibrosis...</i>	172
4.1.2. <i>Alveolar epithelial cells produce FX.....</i>	174
4.1.3. <i>The lung is capable of generating the mature form of FX.....</i>	176
4.1.4. <i>Potential mechanisms leading to increased expression of FX in pulmonary fibrosis.....</i>	176
4.1.5. <i>Activation of FX in the extravascular space.....</i>	177
<b>4.2. The role of FXa in fibroblast to myofibroblast differentiation.....</b>	<b>179</b>
4.2.1. <i>FXa-induces the differentiation of lung fibroblasts to myofibroblasts.....</i>	179

---

4.2.2. FXa exerts its stimulatory effects on myofibroblast differentiation via PAR <sub>1</sub> coupling to Gα <sub>q</sub> .....	182
4.2.3. FXa induces myofibroblast differentiation via a TGF-β <sub>1</sub> -dependent mechanism in vitro.....	185
4.2.4. In vivo evidence for FXa-induced myofibroblast differentiation via a TGF-β and TSP-1 dependent mechanism.....	188
4.3. Role of FXa in bleomycin-induced lung injury and fibrosis.....	188
4.3.1. ZK 807834.....	188
4.3.2. ZK 807834 blocks the profibrotic effects of FXa in vitro.....	189
4.3.3. Effect of ZK 807834 on lung collagen accumulation in bleomycin-induced lung injury and fibrosis.....	190
4.3.4. Possible mechanisms by which FXa inhibition by ZK 807834 may have afforded protection in the bleomycin model of lung injury and fibrosis.....	191
4.3.5. Problems encountered during the assessment of ZK 807834 in the bleomycin model of lung injury and fibrosis.....	193
4.4. Conclusions and implications.....	193
4.4.1. A novel origin for procoagulant activity in the lung.....	194
4.4.2. FXa is a novel pathogenic factor in fibrotic lung disease.....	194
4.4.3. Possible therapeutic implications for antifibrotic compounds.....	195
4.5. Future studies.....	198
a) The role of local FX in mediating the fibrotic response to bleomycin-induced lung injury.....	199
b) Mechanisms leading to increased expression of FX in the fibrotic lung	199
c) The role in mediating EMT and fibrocyte recruitment.....	199
d) Differential downstream signaling effects of FXa and thrombin.....	200
e) Mechanism of protection of FXa inhibition in bleomycin-induced lung injury and fibrosis.....	200
Chapter 5: References.....	201
Publications arising from this thesis.....	231
Academic awards arising from this thesis.....	231



---

## LIST OF FIGURES

Figure 1.1. Photomicrographs of histological sections taken from normal human and IPF lung.....	22
Figure 1.2. Cytokine balance Th1 Vs Th2.....	28
Figure 1.3. Potential origins of myofibroblasts in fibrotic lung disease.....	36
Figure 1.4. TGF- $\beta$ , Smad signalling pathway.....	40
Figure 1.5. Activation of TGF- $\beta$ , by TSP-1.....	43
Figure 1.6. The coagulation cascade.....	48
Figure 1.7. Transcriptional profiling of bleomycin-induced lung injury and fibrosis.....	54
Figure 1.8. Processing and activation of FX.....	55
Figure 1.9. Activation of proteinase-activated receptors.....	58
Figure 1.10. Major PAR <sub>1</sub> G-protein coupled signalling pathways.....	62
Figure 3.1. FX gene expression in bleomycin-induced lung injury and fibrosis...	96
Figure 3.2. FX mRNA levels in whole lung homogenates at 7, 14 and 21 days post bleomycin- induced lung injury .....	97
Figure 3.3. FX gene expression in isolated murine fibroblasts and alveolar epithelial cells post-bleomycin- induced lung injury and fibrosis.....	99
Figure 3.4. Immunohistochemical localization of FX/FXa in bleomycin-induced lung injury and fibrosis.....	101
Figure 3.5. FXa levels in BALF following bleomycin instillation.....	102
Figure 3.6. FX mRNA levels in total human fibrotic and donor lung specimens.....	104
Figure 3.7. FX expression in microdissected alveolar septae from human IPF and donor lungs.....	104
Figure 3.8. FX expression in fibroblasts isolated from human donor and fibrotic lung specimens.....	105
Figure 3.9. Immunohistochemical localization of FX/FXa in human IPF.....	107

---

Figure 3.10. Immunohistochemical localization of FX/FXa in human UIP tissue microarrays.....	108
Figure 3.11. FX expression at the mRNA and protein level in human lung epithelial cells in vitro.....	110
Figure 3.12. Effect of bleomycin stimulation on FX mRNA levels in alveolar epithelial cells.....	112
Figure 3.13. FXa induces fibroblast $\alpha$ -SMA expression in a concentration-dependent manner.....	116
Figure 3.14. FXa induces fibroblast to myofibroblast differentiation in a time-dependent manner.....	117
Figure 3.15. Effect of pHALF passage number on FXa-induced $\alpha$ -SMA expression.....	118
Figure 3.16. FXa induces $\alpha$ -SMA expression in human foetal-and mouse lung fibroblasts.....	119
Figure 3.17. FXa and thrombin have similar effects on $\alpha$ -SMA expression.....	121
Figure 3.18. Effect of FXa stimulation on $\alpha$ -SMA gene expression in pHALFs.....	122
Figure 3.19. Effect of FXa on $\alpha$ -SMA fibre assembly in pHALFs at 36 hours.....	124
Figure 3.20. Effect of FXa on $\alpha$ -SMA fibre formation in pHALFs at 96 hours.....	125
Figure 3.21. Effect of FXa and thrombin on $\alpha$ -SMA fibre formation in HFL-1 at 36 hours.....	126
Figure 3.22. The effect of hirudin on FXa-induced $\alpha$ -SMA expression in pHALFs	129
Figure 3.23. The effect of antistasin core peptide on FXa-induced $\alpha$ -SMA protein induction.....	131
Figure 3.24. Expression of PAR <sub>1</sub> and PAR <sub>2</sub> in pHALFs, HFL-1 and MLFs.....	132
Figure 3.25. The effect of TFLLR-NH <sub>2</sub> , SLIGKV-NH <sub>2</sub> and FTLLR-NH <sub>2</sub> on FXa-induced $\alpha$ -SMA protein induction in pHALFs.....	134
Figure 3.26. The effect of TFLLR-NH <sub>2</sub> and FTLLR-NH <sub>2</sub> on FXa-induced $\alpha$ -SMA protein induction in MLFs.....	135
Figure 3.27. Effect of FXa and TGF- $\beta$ on PAR <sub>1</sub> -deficient mouse lung fibroblasts..	136
Figure 3.28. The effect of the highly selective PAR <sub>1</sub> antagonist, RWJ-58259 on FXa-induced $\alpha$ -SMA expression.....	137
Figure 3.29. Effect of PTX on FXa-induced $\alpha$ -SMA expression.....	139
Figure 3.30. Effect of FXa stimulation on $\alpha$ -SMA protein induction in G $\alpha_q$ -deficient mouse lung fibroblasts.....	140

---

Figure 3.31. Effect of the selective $G_{\alpha_q}$ antagonist Q94 on FXa-induced $\alpha$ -SMA induction.....	141
Figure 3.32. FXa does not stimulate TGF- $\beta$ gene expression.....	145
Figure 3.33. FXa-induced luciferase activity is not blockable by 1D11 and SB431542.....	146
Figure 3.34. Effect of TFLLR-NH <sub>2</sub> on TGF- $\beta$ activity.....	147
Figure 3.35. Time course experiment for phosphorylation of SMAD 2/3 by FXa...	148
Figure 3.36. Effect of FXa stimulation on TSP-1 mRNA levels.....	150
Figure 3.37. Effect of FXa on TSP-1 protein level in pHALFs.....	151
Figure 3.38. Effect of TSP-1 blocking peptide on TFLLR-NH <sub>2</sub> -induced TGF- $\beta$ activation.....	152
Figure 3.39. Effect of inhibition of ALK5 activity with SB431542 on FXa-induced $\alpha$ -SMA expression.....	154
Figure 3.40. Effect of inhibition of ALK5 activity with SD-208 on FXa-induced $\alpha$ -SMA expression.....	155
Figure 3.41. Effect of inhibition of ALK5 activity with SB431542 on TGF- $\beta$ -induced $\alpha$ -SMA expression.....	156
Figure 3.42. Effect of inhibition of ALK5 activity with SD-208 on TGF- $\beta$ -induced $\alpha$ -SMA expression.....	157
Figure 3.43. Immunohistochemical localisation of PAR <sub>1</sub> , TSP-1 and $\alpha$ -SMA in IPF.....	158
Figure 3.44. Effect of ZK 807834 on FXa-induced $\alpha$ -SMA expression.....	161
Figure 3.45. Effect of ZK 807834 on FXa-induced fibroblast proliferation in vitro	162
Figure 3.46. Effect of ZK 807834 on mouse body weight in bleomycin-induced lung injury and fibrosis.....	164
Figure 3.47. Effect of ZK 807834 on lung collagen accumulation in bleomycin-induced lung injury and fibrosis.....	165
Figure 3.48. The effect of oropharyngeal instillation of ZK 807834 on lung histopathology in bleomycin-induced lung injury and fibrosis.....	167
Figure 3.49. Effect of administration of ZK 807834 by oropharyngeal instillation in bleomycin-induced lung injury and fibrosis.....	168
 Figure 4.1. Proposed Mechanism underlying the contribution of FX to lung fibrosis.....	 196

---

## LIST OF TABLES

Table 1.1. Clinical classification of ILDs.....	20
Table 1.2. Classification of the idiopathic interstitial pneumonias.....	21
Table 1.3. Mediators of fibroblast function implicated in lung fibrosis.....	25
Table 1.4. TGF- $\beta$ superfamily and their signalling receptors.....	38
Table 1.5. Mediators of TGF- $\beta$ gene induction.....	41
Table 1.6. Proteinase-activated receptors (PARs).....	60
Table 1.7. Pro-inflammatory and pro-fibrotic mediators induced in response to PAR <sub>1</sub> activation.....	68
Table 2.1. Conditions and buffers for the separation of hydroxyproline by reverse-phase HPLC.....	78
Table 2.2. Primers used for real-time RT-PCR.....	81
Table 3.1. Effect of TGF- $\beta$ and TNF- $\alpha$ -stimulation on FX mRNA levels in A549....	112

---

## LIST OF ABBREVIATIONS

$\alpha$ -SMA	Alpha Smooth Muscle Actin
AIP	Acute Interstitial Pneumonia
ALI	Acute Lung Injury
APC	Activated Protein C
ARDS	Adult Respiratory Distress Syndrome
ASN	Antistatin
ATP	Adenosine Triphosphate
BALF	Brochoalveolar Lavage Fluid
BCA	Bicinchoninic Acid
bFGF	Basic Fibroblast Growth Factor
BOOP	Bronchiolitis Obliterans-Organising Pneumonia
bp	Base Pair
BSA	Bovine Serum Albumin
Ca <sup>2+</sup>	Calcium Ions
cDNA	Completmentary DNA
COP	Cryptogenic Organising Pneumonia
CFA	Cryptogenic Fibrosing Alveolitis
CTGF	Connective Tissue Growth Factor
DAG	Diacylglycerol
DMEM	Dulbecco's Modification of Eagle's Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetracetic Acid
EPCR	Endothelial Protein C Receptor
ERK	Extracellular Signal Related Protein Kinase
ET-1	Endothelin-1
FGN	Fibrinogen
FBN	Fibrin
FXa	Coagulation Factor X (active)
Gla	$\gamma$ -Carboxyglutamic Residue
HCL	Hydrochloric Acid

---

HFL-1	Human Foetal Lung Fibroblast Type 1
HPLC	High Pressure Liquid Chromatography
hpy	Hydroxyproline
ICAM-1	Intercellular Cell Adhesion Molceule-1
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-Like Growth Factor-1
IIP	Idiopathic Interstitial Pneumonia
IL	Interleukin
ILD	Interstitial Lung Disease
IPF	Idiopathic Pulmonary Fibrosis
kDa	Kilodalton
Ki	Dissociation Constant
KO	Knockout
LIP	Lymphoid Interstitial Pneumonia
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MLF	Mouse Lung Fibroblasts
MMP	Matrix Mettalloproteinase
mRNA	Messenger Ribonucleic Acid
NBD-CI	7-chloro-4-nitro-2-oxa-1,3-diazole
NCS	Neonatal Calf Serum
NSIP	Non Specific Interstitial Pneumonia
OP	Organising Pneumonia
PAR	Protease-Activated Receptor
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor-AB
pHALF	Primary Human Adult Lung Fibroblast
PKC	Protein Kinase C
PL	Membrane Phospholipid
RGD	Arginine-Glycine-Aspartic Acid
RNA	Ribonucleic Acid
RT	Room Temperature
rTAP	Recombinant Tick Anticoagulant Peptide
SDS	Sodium Dodecyl Sulphate

---

s.e.m.	Standard Error of the Mean
SN-1	Sorting Nexin-1
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TF	Tissue Factor
TFPI	Tissue Factor proteinase Inhibitor
TGF- $\beta$	Transforming Growth Factor $\beta$
TIMP	Tissue Inhibitor of Metalloproteinase
TNF $\alpha$	Tumour Necrosis Factor Alpha
tPA	Tissue-type Plasminogen Activator
UIP	Usual Interstitial Pneumonia
uPA	Urokinase-type Plasminogen Activator

## Chapter 1: Introduction

### 1.1. Pulmonary Fibrosis

Pulmonary fibrosis is a pathological condition that is classically characterised by excessive and disorganised deposition of extracellular matrix (ECM) within the pulmonary interstitium. The main areas affected include the alveolar walls (including the epithelium and capillary endothelium), septae and the perivascular, perilymphatic and peribronchiolar connective tissue. It is generally held that this condition occurs as an aberrant wound healing response to acute or chronic lung injury. It profoundly compromises alveolar gas exchange and hence often leads to premature death. Varying degrees of pulmonary fibrosis in conjunction with inflammation are the hallmark of all interstitial lung diseases (ILDs), which are a heterogeneous group of over 200 different disorders.

Although many of the ILDs share similar clinical, radiographic and sometimes even pathological features, the mechanisms of disease, treatment, prognosis and outcome are often very different. Until recently, classification of this group of diseases has therefore proven to be problematic. Some of the ILDs have known aetiologies including occupational factors (e.g. asbestosis, silicosis), systemic disorders (systemic sclerosis, rheumatoid arthritis) or are drug-induced (bleomycin, nitrofuramide). These can therefore be grouped into similar clinical characteristics (**Table 1.1.**). In contrast, most cases of ILD have no known aetiology and are termed idiopathic interstitial pneumonias (IIPs). In 1969, Liebow and Carrington first proposed a classification of the IIPs into five distinct histopathological categories. However, in 1998 these were reclassified by Katzenstein and Myers into a unifying category. Therefore, patients that presented with interstitial lung disease of unknown cause were diagnosed as having cryptogenic fibrosing alveolitis (CFA) or idiopathic pulmonary fibrosis (IPF). This is no longer correct as more recently, an expert committee, reclassified these diseases into a new classification scheme, which was adopted, by consensus, by the European Respiratory Society and the American Thoracic Society (American Thoracic Society/ European Respiratory Society International multidisciplinary consensus classification of the idiopathic interstitial pneumonias, 2002. These classified the IIPs into seven distinct disease entities which can be distinguished on the basis of clinical, radiological and histological characteristics (**Table 1.2.**).



**Table 1.1. Clinical classification of ILDs** (adapted from (Laurent and Shapiro, 2007))

**Table 1.2. Classification of the idiopathic interstitial pneumonias**

(Table adapted from: American Thoracic Society/ European Respiratory Society International multidisciplinary consensus classification of the idiopathic interstitial pneumonias, 2002)

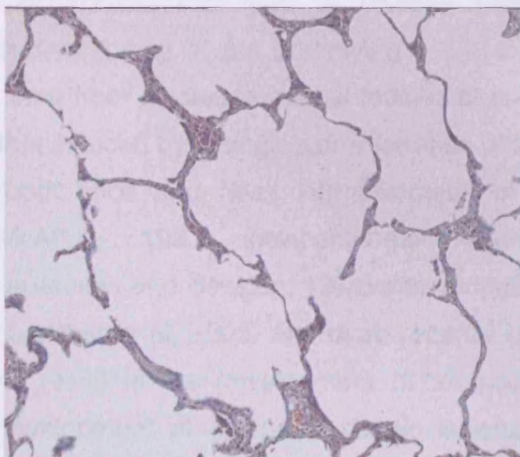
**1.2. Idiopathic Pulmonary Fibrosis (IPF)**

IPF is the most common form of all IIPs, accounting for more than 45% of all cases (American Thoracic Society, 2000). Although epidemiological data on this disease varies in the literature, the number of deaths related to IPF is estimated as 14 times greater than those related to asbestosis with a reported prevalence of approximately 14 to 42.7 cases per 100,000 (Noth and Martinez, 2007), depending on a narrow or wide case definition (Raghu, 2006). It is further estimated that in the U.K. alone, over 1500 people die of this condition per year (Johnston et al, 1990). These numbers are expected to rise further as recorded incidences have increased annually by 11% between the years 1991 and 2003, suggesting that the number of cases is doubling every 8 years (Gribbin et al, 2006). It is more common in men and the elderly and the mean age of onset typically lies between the ages of 67 and 69 years (Coultas et al, 1994). There is no geographical pattern of disease distribution and no racial or ethnic predisposition.

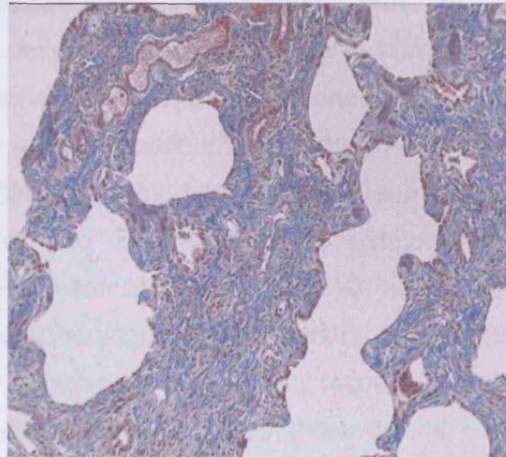
IPF is also the most severe form of all IIPs which always progresses to destruction of the lung parenchyma and thus has a very poor prognosis (Selman et al, 2001). From the onset of symptoms of cough and breathlessness, the medium survival is estimated

as 2.9 years, although the clinical course is often variable with some patients remaining stable for longer periods of time, whilst others experience acute exacerbations leading to rapid progressive respiratory failure and eventual death (Martinez et al, 2005). In addition, IPF appears to be resistant to conventional anti-inflammatory therapies, which are the main line of therapy used in the treatment of all IIDs (reviewed in NHLBI Workshop, American Thoracic Society/ European Respiratory Society International multidisciplinary consensus classification of the idiopathic interstitial pneumonias, 2002). It is therefore very important to distinguish IPF/CFA from other types of IIPs. IPF has a classical, well characterised histological pattern referred to as usual interstitial pneumonias (UIP). Histologically, significant distortion of the lung architecture with fibrosis, honeycombing (enlarged and distorted airspaces resulting from the destruction of normal lung architecture), fibrotic foci (dense areas of proliferating fibroblasts) and mild to moderate inflammation are present (**Figure 1.1.**). Classically, IPF affects both halves of the lung, however the distribution of lesions is very heterogeneous and patchy opacities can be observed on chest x-ray particularly associated with the lower lobes and around subpleural areas. Despite these distinct histological patterns, diagnosing IPF is still proving to be difficult. Normally, IPF lungs have regions of normal lung parenchyma interspersed with regions with a UIP pattern. However, in some cases, a UIP pattern may be seen in one surgical specimen, whilst specimens taken from other lung lobes have an entirely different histological pattern, although the clinical course is characteristic of IPF (Flaherty et al, 2001).

**A. Normal lung**



**B. IPF lung**



**Figure 1.1. Photomicrographs of histological sections taken from normal human lung (A) and IPF lung (B), stained with Martius Scarlet Blue** In the IPF lung alveolar architecture appears distorted due to excessive collagen deposition (shown here in blue) within the alveolar interstitium (Images kindly provided by Dr Robin McAnulty, University College London).

Although the aetiology for IPF is unknown, several risk factors have been proposed. Some theories suggest that damaging environmental agents and occupational factors such as farming, hairdressing and stone-cutting may play a role, although no clear link has yet been established (Baumgartner et al, 2000; Iwai et al, 1994). Cigarette smoking is a risk factor for IPF, however in patients with established IPF, smoking appears to improve disease prognosis (Baumgartner et al, 1997). Viral infections, such as those caused by Epstein-Barr virus and cytomegalovirus, have also been proposed to cause IPF, although no conclusive evidence has yet been provided (Tang et al, 2003). Medication such as anti-depressants may also play a role (Hubbard et al, 1998). Three percent of IPF patients may have familial causes suggesting that genetic factors are involved. Thus, gene polymorphisms in surfactant protein C (Lawson et al, 2004), transforming growth factor beta-1 (TGF- $\beta_1$ ), tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin-1-receptor antagonist (IL-1R), interleukin-6 (IL-6) and in genes with major histocompatibility complex loci have been identified. Nevertheless, only a small proportion of those exposed to known risk factors develop actual disease, suggesting that IPF is multi-factorial and that varying degrees of susceptibility exist (Verleden et al, 2001).

### 1.3. Pathogenesis of Pulmonary Fibrosis

The pathogenesis of pulmonary fibrosis is complicated, and although important advances have been made in the past decade, the specific molecular and cellular mechanisms that lead to disease progression are still unknown. Most of our understanding of the underlying cell-and molecular pathways leading to fibrosis has come from studies in animal models of pulmonary fibrosis, the commonest of which is that induced by a single administration of the anti-neoplastic drug bleomycin to rodents (both mice and rats). Administration of this agent by intratracheal (Laurent and McNulty, 1983), intraperitoneal (Adamson and Bowden, 1974), subcutaneous (Adamson and Bowden, 1974) intravenous (Adamson and Bowden, 1974), aerosolised (Gunther et al, 2003) and more recently by oropharyngeal route (Lakatos et al, 2006) all result in the development of an acute inflammatory response followed by the development of extensive fibrotic lesions which usually subside three weeks post-injury. Interestingly, similar to the human disease, the extent and the severity of fibrosis following bleomycin injury in experimental animals is dependent on the genetic background of the particular strain. Whilst some mouse strains show progressive fibrosis (e.g. C57/Bl6), others (e.g. Balb/c) appear to be resistant to this process (Bonniaud et al, 2004). Although this model has been much criticised due to its known

aetiology, its resolving nature and the lack of restrictive physiology as observed in patients with pulmonary fibrosis it has nevertheless helped the discovery of numerous basic mechanisms that are likely to be important in the pathogenesis of human pulmonary fibrosis.

It is generally held that one of the first events that occur in the development of pulmonary fibrosis is injury to the epithelium and endothelium, from the, as yet, unidentified stimuli. Evidence for this was provided by ultrastructural analysis of IPF lung tissues which demonstrated severe loss of type II alveolar epithelial cells, denudation of basement membranes as well as degeneration of the basement membrane of the capillary endothelium (Corrin et al, 1985).

In a normal healing response tissue injury triggers an acute inflammatory response which mediates the migration, proliferation and activation of mesenchymal cells, ensuring limited deposition of ECM into the wound space. Subsequently, myofibroblast contraction ensures wound closure after which these cells undergo apoptosis. Finally, epithelial cell proliferation promotes repopulation of the basement membrane. In pulmonary fibrosis it is hypothesised that extensive, multiple or persistent insults over time result in an abnormal response to tissue injury, where wound healing is grossly dysregulated (reviewed in (Keane et al, 2006)). Continuous loss of basement membrane integrity results in inflammation and the inability of the alveolus to re-epithelialise. In addition, hyperplastic and proliferating type II alveolar epithelial cells that attempt to repair the disrupted basement membrane (Kasper and Haroske, 1996) produce a number of mediators that drive exaggerated proliferation, activation and migration of underlying fibroblasts into the provisional matrix. This results in the formation of fibrotic foci, consisting of fibroblasts that appear to be resistant to apoptosis, which continuously occupy alveolar airspaces and promote the excessive deposition of ECM leading to irreversible loss of lung function. Interestingly, recent evidence by Cool et al demonstrates that fibrotic foci are interconnected, forming a continuous fibrotic reticulum which suggests that individual foci may not arise from discrete sites of lung injury (Cool et al, 2006).

As already eluded to, a host of mediators including growth factors, chemokine, coagulation proteinases, proteinase inhibitors and cytokines have been shown to influence fibroblast function in the fibrotic lung, some of which are now being pursued in clinical trials (reviewed in (Scotton C.J. and Chambers, 2007)); The major mediators are summarised in **Table 1.3.**

Mediators	References
<b>Chemokines</b>	
CXCL12	(Phillips et al, 2004;Agostini and Gurrieri, 2006)
CCL2/MCP-1	(Gharaee-Kermani et al, 1996)
<b>Cytokines</b>	
IL-1 $\beta$	(Postlethwaite et al, 1984;Singh et al, 1988;Elias et al, 1990)
IL-4	(Wallace et al, 1995;Gillery et al, 1992)
IL-13	(Kolodsick et al, 2004)
TNF- $\alpha$	(Vilcek et al, 1986;Elias et al, 1990)
ET-1	(Cambrey et al, 1994;Dawes et al, 1996)
IFN- $\gamma$	(Gurujeyalakshmi and Giri, 1995;Hunninghake et al, 1986;Elias et al, 1990)
PGE <sub>2</sub>	(Saltzman et al, 1982)
<b>Growth factors</b>	
TGF- $\beta_1$	(Raghow et al, 1987;Willis et al, 2005)
CTGF	(Frazier et al, 1996)
PDGF	(Butt et al, 1995;Bonner, 2004)
IGF-1	(Phillips et al, 1987;Goldstein et al, 1989)
<b>Coagulation proteinases</b>	
Thrombin	(Chan et al, 1998;Chambers et al, 1998;
Factor Xa	Blanc-Brude et al, 2005)

**Table 1.3. Mediators of fibroblast function implicated in lung fibrosis**

These pro-inflammatory and pro-fibrotic mediators act via autocrine and paracrine mechanisms and are released from a variety of cell types including fibroblasts, inflammatory cells, endothelial cells, smooth muscle cells and platelets, although activated alveolar epithelial cells are thought to be a major source of these mediators in the fibrotic lung (Kasper and Haroske, 1996). Pro-fibrotic mediators which promote the pathogenesis of pulmonary fibrosis, include factors such as TGF- $\beta$ , TNF- $\alpha$  and IL-13. Various anti-fibrotic mediators which are thought to counteract fibrotic processes have also been described, including IFN- $\gamma$ , PGE<sub>2</sub> and the antioxidant glutathione and may be deficient in the IPF lung leading to an imbalance in pro-fibrotic and anti-fibrotic factors.

### **1.3.1. The role of inflammation in the pathogenesis of IPF**

The question as to what role inflammation plays in the process of pulmonary fibrosis, particularly in the context of IPF, is highly controversial and subject of much debate. On the basis of historical studies of IPF biopsy material, inflammation was implicated as the major pathogenic mechanism involved (Selman et al, 2001; Ward and Hunninghake, 1998). This was based on the idea that persistent inflammation of the epithelial and vascular compartments leads to the loss of alveolar wall integrity and thus fibrotic changes. IPF was therefore regarded as a chronic inflammatory disorder and therapy was aimed at targeting inflammatory pathways (Ward and Hunninghake, 1998). Unfortunately, none of the commonly used anti-inflammatory or immunosuppressive drugs such as prednisolone, azathioprine or cyclophosphamide, have shown any beneficial effect in slowing-down the progression of this disease. However, there are currently no effective therapies so that these drugs are still being prescribed as main line therapy for this condition (Raghu, 2006).

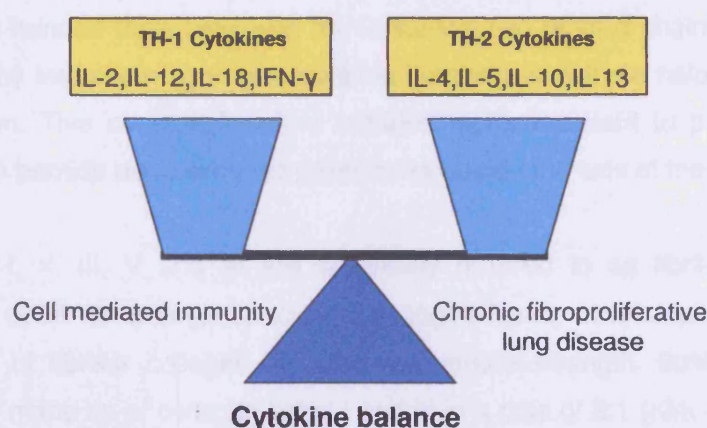
More recently, an alternative hypothesis has thus been proposed suggesting that IPF is the result of an aberrant interaction between the epithelium and mesenchyme, with little or no involvement of an inflammatory component (Selman et al, 2001). This theory is supported by the observation that fibroblast foci in IPF form in areas directly underneath the damaged epithelium, representing the 'leading edge' of the fibrogenic processes. The observation that similar degrees of inflammation exist both in the early as well as in the late stages of disease further supports this concept (Gauldie, 2002). In addition, over-expression of the potent pro-fibrotic mediator TGF- $\beta$ , leads to progressive fibrosis and remodelling in mice, without any significant inflammatory involvement (Kolb et al, 2001). Similarly, mice deficient in the  $\alpha_v\beta_6$  integrin are unable to effectively activate TGF- $\beta$  and thus develop marked inflammation post bleomycin-induced lung injury but this response fails to progress to fibrosis (Munger et al, 1999).

This theory has been at the centre of an active debate. Opponents argue that this theory only provides a 'snapshot' view of the disease and does not look at the natural history of IPF and disregards the common observation that tissue injury is always followed by an inflammatory response (Strieter, 2002). In addition, adenoviral expression studies such as those performed with TGF- $\beta$  may be criticisable, as this cytokine is overexpressed at unphysiological concentrations (Strieter, 2002). Whilst anti-inflammatory therapy may not help for end-stage disease pathology, it is argued that a beneficial effect can not be ruled out at early stages of IPF where inflammation may play a role. Evidence for this has come from studies examining the histopathological variability of surgical tissue sections of patients with IIP (Flaherty et al, 2001). Whilst chronic inflammation is a major feature of inflammatory non-specific interstitial pneumonia (NSIP) this is not the case for IPF. However in 26% of patients both states co-existed in the same biopsy specimens, raising the possibility that the natural history of UIP may begin with the development of NSIP, which when left untreated, leads to progressive UIP in the later stages. Further strong evidence for an involvement of the inflammatory system has come from studies using microarray analysis to compare gene expression patterns of lung tissues taken from patients with IPF versus normal donor lungs (Zuo et al, 2002). The authors found four categories of genes highly expressed in patients with IPF, amongst which were genes encoding pro-inflammatory cytokines, chemokines and antioxidants, but also genes encoding amyloid, which are all associated with chronic inflammatory disorders (Strieter, 2005). Furthermore, no difference was observed in the gene expression profiles obtained for patients with IPF compared to that obtained from patients with NSIP (Rosas and Kaminski, 2007) suggesting that inflammation might well be a major part of IPF.

A unifying hypothesis has been suggested that combines both of the above theories. This theory proposes that acute inflammation precedes the chronic fibrotic process, however inflammation is necessary but not sufficient for fibrogenesis to occur (Sheppard, 2001b). Evidence for this has been provided by a study performed by Kolb and colleagues where transient overexpression of IL-1 $\beta$  lead to an acute inflammatory response that evolved into pulmonary fibrosis (Kolb et al, 2001). One potential interpretation of this result is that in this system IL-1 $\beta$ -mediated inflammation induces the defective cross-talk between mesenchymal cells and epithelial cells leading to failure of normal re-epithelialization and re-endothelialization. Subsequent fibrosis is then driven by TGF- $\beta$ , persistent expression of which occurs in the context of IL-1 $\beta$ -induced inflammation.



Other theories suggest that multiple injuries lead to an imbalance in Th-1/Th-2-type cytokines. Analysis of lung tissue and bronchoalveolar lavage fluid (BALF) samples taken from patients with IPF revealed an inflammatory pattern characteristic of a TH-2 immune response, as demonstrated by an increase in eosinophils, mast cells and cytokines such as IL-4, IL-5, IL-10 and IL-13 (Rottoli et al, 2005). It was therefore suggested that a local imbalance in TH-1/TH-2-type cytokines favours a Th-2 profile (Antoniou et al, 2007; Strieter, 2002) which directly contributes to the fibrogenic process (**Figure 1.2.**). A link between a TH-2 type profile and fibrosis has now been well established in animal models of pulmonary fibrosis, although a functional link in human IPF is still lacking (Thannickal et al, 2004). IFN- $\gamma$ , which is one of the major cytokines implicated in the modulation of TH-1/TH-2 imbalance, has thus been proposed as a promising target in IPF. However, recent clinical trials assessing the therapeutic potential of IFN- $\gamma$  in IPF, were largely disappointing but showed a glimpse of hope (reviewed in (Scotton C.J. and Chambers, 2007)).



**Figure 1.2. TH-1/TH-2 Cytokine balance in pulmonary fibrosis**

Lung fibrosis involves a shift in the local balance of TH-1/TH-2-type cytokines in favour of a TH-2 profile. TH-2 cytokines such as IL-4 and IL-13 promote fibrosis by stimulating fibroblast collagen synthesis whilst TH-1 cytokines such as IFN- $\gamma$  may inhibit fibroblast function and collagen synthesis and are thus anti-fibrotic.

## 1.4. Extracellular matrix deposition in the fibrotic lung

As discussed in the previous section, a number of mechanisms may lead to the development of pulmonary fibrosis. However, the excessive deposition of collagen and other ECM components within the pulmonary interstitium remain the hallmarks of this disorder. The next section will focus on the distribution and metabolism of collagen in the normal and fibrotic lung.

### 1.4.1. Collagen structure

The collagens are a family of 28 closely related extracellular matrix proteins, each comprising a characteristic triple helix consisting of three polypeptide chains of unique but homologous amino acid sequence (Pace et al, 2003)). At least 11 different collagen subtypes are known to be present in the lung (reviewed in (Chambers, 1997)). Collagen subtypes can be classified into homotrimers composed of three identical  $\alpha$ -chains, (e.g. collagen III) or heterotrimers consisting of two or three different  $\alpha$ -chains (e.g. collagen I or collagen VI respectively). Each  $\alpha$ -chain contains a high proportion of glycine residues, which occur in a regularly repeating triplet, Gly-X-Y, where approximately every third X is proline and every third Y is hydroxyproline (Miller, 1985). Each individual  $\alpha$ -chains forms a left handed polyproline helix and further assembles into a right handed triple helix with the remaining two other  $\alpha$ -chains. This structure is stabilised by interchain hydrogen bonding resulting in a triple helix with a coiled-coil confirmation. This structure renders collagen highly resistant to proteolysis as each glycine and peptide bond is buried within or alongside the axis of the helical structure.

Collagens I, II, III, V and XI are classically referred to as fibril-forming interstitial collagens, which form large bundles of collagen fibres, several  $\mu\text{m}$  in diameter. The major role of fibrillar collagen is to provide tensile strength. 90% of the total lung collagen is made up of collagen types I and III in a ratio of 2:1 (Kirk et al, 1984). These collagens are widely distributed throughout the pulmonary interstitium and are believed to be crucial in providing structural integrity in an organ which contains intricate and extensive networks of airspaces and capillaries. Fibroblasts are thought to be a major source of these types of collagen. However, other cell types, including alveolar epithelial cells, endothelial cells and smooth muscle cells have also been shown to produce these collagen types. The major non-fibrillar collagen found in the lung is collagen type IV which serves as an important structural component of the alveolar and capillary basement membrane.

Other important collagen types present in the lung include collagen type V which is mainly found in the basement membrane and pulmonary interstitium, type VI which forms fine filaments with collagens type I and III and collagen type II which is only present in cartilage and is therefore particularly abundant in the large airways. Collagen Types IX and XI are similarly distributed with Type II collagen in the trachea and large airways (reviewed in (Chambers, 1997)).

#### **1.4.2. Collagen deposition in a normal and fibrotic lung**

In the normal lung collagen exists in a dynamic equilibrium where synthesis and degradation are highly regulated. Early studies in our laboratory revealed that as much as one tenth of the total collagen in the lung of a young adult mammal is synthesised and degraded per day (McAnulty and Laurent, 1987). It is generally held that in fibrotic lung disease this balance is disturbed thus leading to increased deposition of collagen (McAnulty and Laurent, 1987). This excessive and disorganised deposition of collagen results in gross thickening of the normally thin alveolar interstitium leading to the destruction and remodelling of the lung architecture and progressive loss of alveolar function.

In early fibrosis, several reports have observed an imbalance in the ratio of type III to type I collagen, whereby the former is thought to increase disproportionately (Kirk et al, 1984). In contrast, once fibrosis is established higher levels of collagen I versus III are observed. However, it is generally held that excessive collagen production rather than a change in the ratio of different types of collagen is important in fibrotic lung disease. For example, increased procollagen I gene expression as measured by *in situ* hybridisation has been documented in patients with IPF (Broekelmann et al, 1991). In addition, elevated levels of procollagen peptides (collagen cleavage products) have also been measured in BALF taken from patients with pulmonary fibrosis (Harrison et al, 1991). Similarly, in animal models of pulmonary fibrosis, such as that induced by bleomycin, increases in expression of the procollagen genes COL1A1, COL1A2 and COL3A1 have been reported (Zhang et al, 1994b; Shahzeidi et al, 1994). In contrast, a decrease in collagen degradation in the fibrotic lung has also been documented. For example, decreased collagenolytic activity has been reported in the lungs of IPF patients (Selman et al, 1986). Further evidence for a non-degradative state in the fibrotic lung has come from studies examining the expression of matrix metalloproteinases (MMPs), important in the degradation of matrix proteins and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). These

studies have shown that TIMPs are upregulated to a greater extent than MMPs in the fibrotic lung aiding persistent matrix deposition (Selman et al, 2000).

Excess collagen is deposited in fibrotic foci and the presence and persistence of fibrotic foci is a key element in the histopathologic diagnosis of IPF and correlates with disease progression and decreased survival of affected individuals (King, Jr. et al, 2001). Thus mechanisms that regulate the deposition of collagen by fibroblasts are the main determinants of disease progression. These include mechanisms that regulate collagen synthesis rates in fibroblasts at a per cell basis by influencing procollagen gene transcription, translation and posttranslational modification. Similarly, collagen degradation in a fibroblast can be modulated by regulating the rate of procollagen mRNA degradation. Additional mechanisms that lead to an increase in the fibroblast cell population in the lung including chemotactic attraction to the site of injury and mitogenesis of fibroblasts. Finally, fibroblasts may undergo phenotypic modulation and change into a myofibroblast phenotype that promotes excessive collagen deposition and thus favours fibrogenesis. The work in this thesis has particularly focused on the role of the myofibroblast in pulmonary fibrosis and the next section will describe this cell phenotype in more detail.

## **1.5. The myofibroblast and pulmonary fibrosis**

### ***1.5.1. Discovery and origin of the myofibroblast***

The myofibroblast was first described 30 years ago as a major cell type involved in the wound healing process. Studies performed by Gabbiani and Majno examined an experimental wound healing model by electron microscopy and observed the transient appearance and disappearance of the myofibroblast within granulation tissue (reviewed in (Desmouliere et al, 2005)). This particular cell phenotype exhibited smooth muscle cell-like features, including bundles of microfilaments, an abundance of rough endoplasmic reticulum and irregular shaped nuclei and it was therefore proposed that the myofibroblast may be responsible for the force generation resulting in wound contraction and connective tissue retraction that is observed during wound healing (Gabbiani et al, 1972). The term myofibroblast was therefore proposed for this plastic and contractile fibroblast phenotype. Extensive work later showed that the myofibroblast was important in a number of biological processes and was not only present in normal tissues, where force generation was needed but also in pathological conditions associated with aberrant wound healing, excessive contraction and extracellular matrix deposition, such as hypertrophic scars, scleroderma, Dupuytren's

disease (Hinz, 2007) as well as fibrotic conditions of the heart (Brown et al, 2005) liver, kidney (Desmouliere et al, 2003) and lung (Adler et al, 1989). This led to the concept that following tissue injury, differentiation of fibroblasts into the myofibroblast phenotype contributes to tissue repair and regeneration, but in pathological states it promotes the severe impairment of organ function through excessive contraction and extracellular matrix deposition.

The differentiation of fibroblasts into the myofibroblast phenotype is a complex process that follows a highly regulated chain of events (Hinz et al, 2007). In normal non-pathological environments fibroblasts express little actin-dependent cell-cell and cell-matrix contacts and their synthetic rate in ECM components is low (Tomasek et al, 2002). Following tissue injury, locally produced cytokines promote the migration of fibroblasts into the wound space and increase their production of ECM components. Mechanical tension arising from disruption of normal tissue integrity further induces the assembly of contractile stress fibres containing cytoplasmic actins. This intermediate fibroblast phenotype is termed the protomyofibroblast (Desmouliere et al, 2005). The acquisition of stress fibres enables this cell type to connect to ECM components through integrin-containing cell matrix junctions and to other cells via cell-cell adherens junctions (Hinz et al, 2004). This cell type also upregulates the production of cellular fibronectin, a variant of fibronectin usually present during development and absent in normal adult connective tissue (Serini et al, 1998). Protomyofibroblasts are particularly observed in tissue culture where rigid plastic substrate generates the necessary tension for the induction of this cell phenotype (Hinz, 2006). *In vivo*, this intermediate phenotype proceeds to differentiate into the myofibroblast which is characterised by the *de novo* expression of the mechanosensitive protein alpha smooth muscle actin ( $\alpha$ -SMA) which is recruited to stress fibres under influence of high tension (Goffin et al, 2006).  $\alpha$ -SMA is therefore considered to be the most significant marker of myofibroblastic cells, although other proteins such as smooth muscle myosin heavy chain, desmin, intermediate filament proteins and caldesmon are also expressed (Hinz et al, 2001; Cogan et al, 2002; Sappino et al, 1990; Chambers et al, 2003). Myofibroblasts are highly synthetic for collagen and other ECM components and the expression of  $\alpha$ -SMA correlates with increased contractility of this cell phenotype (Hinz et al, 2001). The exact mechanism of how increased contractility is achieved is still unknown although the N-terminal region of  $\alpha$ -SMA, Ac-EEED, which is specific to this isoform of actin has been implicated. Evidence for this was provided by studies where micro-injection of synthetic Ac-EEED peptide into myofibroblastic cells caused the disappearance of  $\alpha$ -SMA protein as detected by immunolocalisation and further

reduced the ability of myofibroblasts to contract *in vitro* as well as *in vivo* (Hinz et al, 2002).

### **1.5.2. The role of myofibroblast in pulmonary fibrosis**

The presence of myofibroblasts in tissues taken from patients with pulmonary fibrosis, as well as in animal models of the disease, has been documented for some time (Adler et al, 1989; Mitchell et al, 1989; Kuhn, III et al, 1989; Pache et al, 1998). It was hypothesised that this distinct fibroblast phenotype may contribute to the development and progression of fibrosis. Studies showing increased collagen synthetic capacity of granulation tissue myofibroblasts and their localisation to fibroblastic foci during active matrix deposition strongly favoured such a role (Kuhn and McDonald, 1991). This was further supported by the finding that myofibroblasts were able to synthesise other extracellular matrix components such as vitronectin, fibronectin and laminin, the deposition of which is also an important feature of active disease (Kuhn and McDonald, 1991). The first direct evidence for the myofibroblast as a key source of collagen production in the fibrotic lung came from *in situ* hybridisation studies that confirmed colocalisation of collagen type I mRNA with  $\alpha$ -SMA immunostaining in fibrotic foci of tissue sections taken from the pulmonary fibrosis model induced by bleomycin (Zhang et al, 1994b). This finding was supported by the observation that the appearance and disappearance of myofibroblasts in fibrotic foci correlated with the development and subsequent resolution of active fibrosis in experimental pulmonary fibrosis and that in human disease myofibroblast persistence associated with progression of fibrosis (Zhang et al, 1994b; Kuhn and McDonald, 1991).

The mechanisms leading to an increased ECM synthetic capacity of myofibroblasts are however less well understood. One theory suggests that the myofibroblast undergoes phenotypic changes that permanently 'reprogram' this cell to promote fibrogenesis. Evidence for this has come from studies analysing phenotypic differences between fibroblasts isolated from normal lungs and fibroblasts/myofibroblasts isolated from fibrotic lungs. These studies showed that fibroblasts/myofibroblasts isolated from IPF lungs exhibited an increased synthetic capacity for collagen and other ECM proteins in response to pro-fibrotic mediator stimulation compared to fibroblast isolated from healthy lungs (Hetzl et al, 2005). These fibroblasts/ myofibroblasts also exhibit an enhanced migratory phenotype (Suganuma et al, 1995), increased production of pro-fibrotic mediators (Moodley et al, 2003), and increased contractility of collagen matrices (Miki et al, 2000) and appear to be more resistant to apoptosis (Ramos et al, 2001; Moodley et al, 2004). Similarly, they have been reported to produce less

antifibrotic factors, such as cyclooxygenase-2-derived PGE<sub>2</sub> (Keerthisingam et al, 2001; Wilborn et al, 1995) and are less responsive to the inhibitory effects of PGE<sub>2</sub> on fibroblast proliferation (Mio et al, 1992). However, these studies are not conclusive primarily because they have produced conflicting results. For example, fibroblasts isolated from fibrotic lungs have been shown to have higher proliferative capacities when compared to those isolated from normal donor lung, however, in more advanced stages of the disease these fibroblasts appear to proliferate at lower rates (Raghu et al, 1988; Hetzel et al, 2005). It is also argued that isolated cells may not provide a true representation of pathology as they may undergo phenotypic modulation when grown in *in vitro* culture systems where the cellular microenvironment is very different to the *in vivo* situation.

Whilst there is no dispute that myofibroblasts display an increased synthetic capacity, it is less well established whether the contractile properties of myofibroblasts, which have convincingly been shown in experimental wound healing models, contribute significantly to the pathophysiology of fibrosis. Increased contractility of collagen gels *in vitro* by fibroblasts/myofibroblasts isolated from rat lungs following bleomycin-challenge correlates with an increase in  $\alpha$ -SMA expression (Zhang et al, 1996). Indirect evidence for a role of myofibroblasts in contraction and connective tissue remodelling *in vivo* is reflected in the pathological stiffness of lungs of patients with pulmonary fibrosis (reviewed in (King, Jr. et al, 2001)). However, no direct link has yet been established between increased contractility and decreased lung compliance observed in the fibrotic lung and the expression of  $\alpha$ -SMA positive myofibroblasts *in vivo*.

It has also been suggested that myofibroblasts play a role in the inflammatory process that may accompany fibrotic lung disease and thus potentiate its progression (reviewed in (Phan et al, 1999)). Myofibroblasts represent a significant source of growth factors (Finlay et al, 2000), cytokines (Phan et al, 1999), chemokines (Zhang et al, 1994a) and also reactive oxygen species (Thannickal et al, 1998) and express markers typical of immune cells such as CD40 (Brouty-Boye et al, 2000). In addition, they express adhesion molecules such as intracellular adhesion molecule -1 (ICAM-1) and vascular cell adhesion molecule (VCAM) which enable the docking of inflammatory cells to their cell surface (Burns et al, 1996; Crowston et al, 1997). This has lent credence to the theory that myofibroblasts may modulate the environment of acute resolving inflammation and drive the change to a chronic, persistent inflammation implicated in IPF (Buckley et al, 2001).

Finally, myofibroblast differentiation is thought to play a part in alveolar epithelial cell apoptosis, denudation of basement membrane and retardation of epithelial regeneration (Waghray et al, 2005) thought to contribute to the pathogenesis of pulmonary fibrosis (**see Section 1.3.**). Thus the myofibroblast is not only important in ECM deposition but also promotes inflammation and contributes to the aberrant wound healing process implicated in the pathogenesis of pulmonary fibrosis.

### **1.5.3. Origin of myofibroblasts**

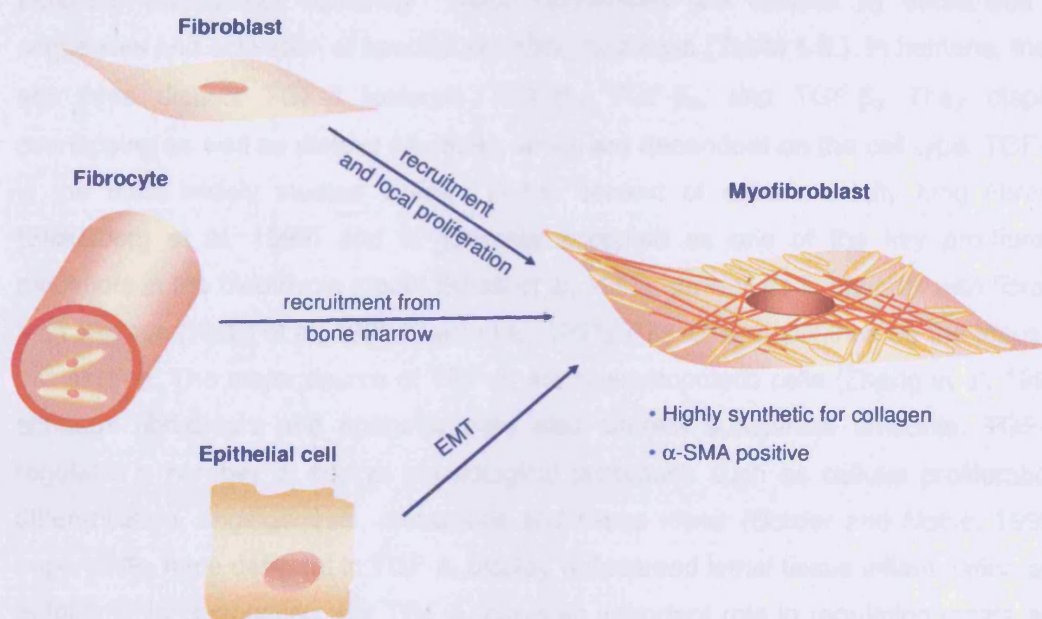
Current opinion suggests that lung myofibroblasts have at least three possible origins although the relative contribution of each of these pathways in pulmonary fibrosis is currently unknown. Based on cytoskeletal phenotype, tissue localization and *in vitro* studies, it is traditionally believed that myofibroblasts are solely derived from resident mesenchymal cells, particularly from peribronchial and perivascular adventitial fibroblasts as well as pericytes and smooth muscle cells (Zhang et al, 1994c; Chamley et al, 1977), the population of which expands in response to stimuli secreted by inflammatory and epithelial cells during the fibrogenic process. In support of this theory, *in vitro* studies have provided biochemical and morphological evidence for a fibroblastic origin (Oda et al, 1988) including the ability of isolated fibroblast to differentiate into the myofibroblast phenotype in response to cytokine stimulation *in vitro* (Serini and Gabbiani, 1999). More recently, although controversial, new studies have suggested additional sources of myofibroblasts derived from transdifferentiation of adjacent epithelial cells (Willis et al, 2005) as well as from circulating fibrocytes and other bone marrow derived progenitor cells ((Abe et al, 2001; Phillips et al, 2004), **Figure 1.3.**).

Fibrocytes were originally identified by Bucala *et al* in 1994 as Collagen I<sup>+</sup>/CD34<sup>+</sup>/CD45RO<sup>+</sup> cells which are likely derived from haematopoietic stem cells (Bucala et al, 1994). While initially these cells were only described in the context of wound healing they have since been found to participate in granuloma formation, antigen presentation and various fibrosing disorders (reviewed in (Gomperts and Strieter, 2007)). Recent studies using bone marrow chimeric mice where bone marrow-derived cells were tracked through their expression of specific marker proteins, have provided evidence that these cells may represent an additional source of fibroblasts in the lung (Hashimoto et al, 2004; Brocker et al, 2006). Evidence for a pathogenic role for fibrocytes in pulmonary fibrosis has recently been provided by studies demonstrating that blockade of fibrocyte recruitment is protective in experimentally-induced pulmonary fibrosis (Moore et al, 2005; Phillips et al, 2004). However, it remains to be determined if



these cells contribute to the excessive collagen deposition seen in IPF or if they have alternative roles such as serving as mediators of a pro-fibrotic milieu.

A potential third cellular source are epithelial cells that undergo transdifferentiation to form fibroblasts and hence myofibroblasts by a process termed epithelial-mesenchymal transition (EMT). The concept of EMT has been recognized for more than twenty years and has been a major focus of research in the field of kidney fibrosis. It is a fundamental process, whereby epithelial cells are thought to lose their characteristic markers, such as E-cadherin and zona occludens (ZO)-1, and acquire mesenchymal markers such as fibroblast specific protein (FSP)-1 and  $\alpha$ -SMA (reviewed in (Willis et al, 2006). Increasing evidence has now emerged supporting a role for EMT in IPF both *in vitro* and *in vivo* (Willis et al, 2005; Kim et al, 2006). However, the relative contribution of this cell type to the fibrotic process needs further investigation.



**Figure 1.3. Potential origins of myofibroblasts in fibrotic lung disease**

Myofibroblasts are key effector cells in IPF which are highly synthetic in collagen and are characterised by the *de novo* expression of  $\alpha$ -SMA. Myofibroblasts are thought to arise from proliferation and differentiation of resident fibroblasts, recruitment of circulating fibrocytes and from epithelial-mesenchymal transition (EMT).

#### **1.5.4. Regulation of myofibroblast differentiation**

At least three major events in granulation tissue formation have been described that drive the myofibroblast differentiation programme which include mechanical stress (Tomasek et al, 2002), the cytokine TGF- $\beta$  (Desmouliere et al, 1993) and components of the ECM such as the ED-A splice variant of cellular fibronectin (Serini et al, 1998). This thesis has particularly worked on the role of TGF- $\beta$  in myofibroblast differentiation in the context of pulmonary fibrosis. A more extensive description of TGF- $\beta$ , its signalling pathways and its effect on myofibroblast differentiation will thus be discussed here in more detail.

### **1.6. Role of TGF- $\beta$ in myofibroblast differentiation and pulmonary fibrosis**

#### **1.6.1. TGF- $\beta$ function and signalling**

TGF- $\beta$  belongs to a superfamily of more than 42 structurally related polypeptides, which are divided into two subfamilies, the TGF- $\beta$ /activin/nodal subfamily and the BMP (Bone Morphogenic Protein)/GDF (Growth and Differentiation Factor)/MIS (Muellerian Inhibiting Substance) subfamily. These subfamilies are defined by similarities in sequences and activation of specific signalling pathways (**Table 1.3.**). In humans, there are three distinct TGF- $\beta$  isoforms TGF- $\beta_1$ , TGF- $\beta_2$ , and TGF- $\beta_3$ . They display overlapping as well as distinct functions, which are dependent on the cell type. TGF- $\beta_1$  is the most widely studied isoform in the context of experimentally lung fibrosis (Eickelberg et al, 1999) and is generally accepted as one of the key pro-fibrotic mediators in the bleomycin model (Khalil et al, 1989), as well as in patients with fibrotic lung disease (Khalil et al, 1996; Khalil et al, 1991). This isoform will thus be the focus of this section. The major source of TGF- $\beta_1$  are haematopoietic cells (Zhang et al, 1995) although fibroblasts and epithelial cells also secrete substantial amounts. TGF- $\beta_1$  regulates a number of normal physiological processes such as cellular proliferation, differentiation, angiogenesis, metastasis and tissue repair (Border and Noble, 1995). Importantly, mice deficient in TGF- $\beta_1$  display widespread lethal tissue inflammation and autoimmunity suggesting that TGF- $\beta_1$  plays an important role in regulating innate and acquired immunity (Kulkarni et al, 1993). Appropriate levels of TGF- $\beta_1$  are therefore necessary for homeostasis and hence bioavailability of this pluripotent cytokine is highly regulated (**see Section 1.6.2.**).

**Table 1.3. TGF- $\beta$  superfamily and their signalling receptors**

BMP= bone morphogenic protein, GDF= growth and differentiation factor, MIS= muellerian inhibiting substance (table adapted from (Massague and Gomis, 2006))

TGF- $\beta_1$  signals by interacting with three classes of receptors, type I (TGF $\beta$ IR or ALK5), type II receptors (TGF $\beta$ IIR) and type III receptor (TGF $\beta$ IIIR, or betaglycan) (**Table 1.3.**, (Rubtsov and Rudensky, 2007)). The characteristic features of TGF- $\beta$  receptors include a toxin fold in the extracellular ligand-binding domain, a single transmembrane domain and an intracellular serine/ threonine kinase domain, whereas TGF $\beta$ IIR only has a short intracellular domain. TGF $\beta$ IIIR acts as an accessory receptor which binds TGF- $\beta_1$  and recruits TGF- $\beta_1$  to TGF $\beta$ IIR. Once TGF- $\beta_1$  has bound to the TGF $\beta$ IIR, hetero-oligomerization with TGF $\beta$ IR occurs, allowing the constitutively active kinase domain of TGF $\beta$ IIR to phosphorylate the TGF $\beta$ IR kinase domain (see **Diagram 1.4.**). This induces a conformational change of the type I receptor and initiates downstream signalling through the recruitment and phosphorylation of the Smad proteins, specifically Smad2 and Smad3 (receptor associated Smads (R-Smads)). Recruitment of R-Smads to the type I receptor can be facilitated by auxiliary proteins such as Smad Anchor for Receptor Activation (SARA)(Tsukazaki et al, 1998). The phosphorylated Smad 2/3 then associates with Smad4 (co-mediator Smad (co-Smad 4)), the complex translocates to the nucleus where it binds directly to DNA and modulates transcription of a large number of genes ((Rubtsov and Rudensky, 2007)). In contrast, the inhibitory Smad (I-Smad-7) negatively regulates TGF- $\beta_1$  signalling by competing with the R-Smads (Nakao et al, 1999). In addition, cell signalling by activated Smad proteins is

terminated by protein phosphatases and ubiquitin ligases. Thus under continuous TGF- $\beta_1$  stimulation Smads undergo constant receptor-mediated phosphorylation and protein phosphatase-mediated dephosphorylation, shuttling in and out of the nucleus (Inman et al, 2002b).

TGF- $\beta_1$  triggers various Smad-independent pathways including ERK1/2, c-Jun NH<sub>2</sub>-terminal kinase (JNK), p38MAPK, PI3K and Akt and Rho-like GTPases (Derynck and Zhang, 2003) and there is complex cross-talk between these pathways and the Smad pathway. However, TGF- $\beta_1$  signalling via the Smad pathway has been widely implicated in the pathogenesis of pulmonary fibrosis (Hu et al, 2003). In endothelial cells TGF- $\beta_1$  may signal via the additional ALK1 (Smad1/5) pathway. This alternative pathway appears to have opposing effects to the classical ALK5 (Smad2/3) pathway which inhibits endothelial cell proliferation and migration (Lebrin et al, 2004). Signalling via the ALK1 (Smad1/5) pathway is facilitated by endoglin, thus cells lacking endoglin have decreased ALK1-and increased ALK5 signalling and thus do not proliferate (Lebrin et al, 2004).

### **1.6.2. The latent TGF- $\beta_1$ complex**

TGF- $\beta_1$  activity is controlled on a number of levels, namely transcription, mRNA, stability and translation, storage of latent TGF- $\beta_1$ , activation of latent TGF- $\beta_1$  and TGF- $\beta$  receptor expression. Although numerous mediators that induce TGF- $\beta_1$  gene expression have been described (**see Figure 1.4.**), activation of this cytokine appears to be a major rate limiting step in the regulation of its bioavailability (Sheppard, 2001a; Sheppard, 2006). TGF- $\beta_1$  is initially synthesised in its precursor form known as pro-TGF- $\beta_1$  and is subsequently processed intracellularly to yield the mature form of the protein. Cleavage of pro-TGF- $\beta_1$  results in the formation of an inactive complex consisting of TGF- $\beta_1$  which is noncovalently associated to an endogenous inhibitor, the latency associated peptide, LAP (Hyytiainen et al, 2004) It is the association of LAP with TGF- $\beta_1$  that renders this cytokine inactive and unable to interact with its receptor and to induce most of its cellular effects. In almost all cells, including fibroblasts, this complex is further bound by disulphide linkage of LAP to another protein family, called latent TGF- $\beta_1$  binding protein (LTBP) (Todorovic et al, 2005). This binding protein directly cross-links the complex to fibronectin, so that high concentrations of the latent form of this cytokine are bound to the extracellular matrix (Sheppard, 2001a).



**Figure 1.4. TGF- $\beta_1$  SMAD signalling pathway**

TGF- $\beta_1$  signals via the Smad pathway. TGF- $\beta_1$  binds to the TGF $\beta$ RIIR which facilitates TGF- $\beta_1$  binding to TGF $\beta$ RIIR. This leads to recruitment of the type I receptor and hetero-oligomerization of TGF $\beta$ RIIR with TGF $\beta$ IR occurs, allowing the constitutively active kinase domain of TGF $\beta$ IR to phosphorylate the TGF $\beta$ IR kinase domain, which propagates the signal downstream through phosphorylation of Smad2 and Smad3. Smad2 and Smad3 then form a complex with Smad4. The complex translocates to the nucleus, where it activates target genes. The inhibitory Smad (Smad7) negatively regulates TGF- $\beta_1$  signalling by competing with the Smads2 and 3. (Image taken from Cambridge University Press, Expert reviews, 2003)

<b>Mediator</b>	<b>Cell type</b>	<b>Reference</b>
IL-1 $\beta$	Aortic smooth muscle cells, Endothelial cells	(Yue et al, 1994;Phan et al, 1992)
CCL2/MCP-1	Fibroblasts	(Gharaee-Kermani et al, 1996)
PDGF	Chondrocytes	(Villiger and Lotz, 1992)
bFGF	Pituitary cells	(Qian et al, 1996)
Angiotensin II	Cardiac fibroblasts and myofibroblast	(Campbell and Katwa, 1997)
IL-13 in combination with TNF- $\alpha$	Macrophages	(Fichtner-Feigl et al, 2006)
TNF- $\alpha$	Endothelial cells	(Phan et al, 1992)

**Table 1.4. Mediators of TGF- $\beta$  gene induction**

In order for TGF- $\beta_1$  to activate its receptor and induce cell signalling, the cytokine needs to be converted to its active state. This process requires degradation of LAP or alteration of the interaction of LAP with TGF- $\beta_1$ . *In vitro*, numerous stimuli that induce mild protein denaturation have been shown to activate TGF- $\beta_1$ , including extremes of temperature or pH as well as exposure to oxidants and ionizing radiation (Munger et al, 1997). However, the relative significance of these mechanisms *in vivo* remains unknown. *In vivo*, several possible TGF- $\beta_1$  activation mechanisms have been described. Evidence from smooth muscle-cell and endothelial cell co-culture systems suggests that proteolysis by plasmin represents a potential mechanism of activation for TGF- $\beta_1$ . Plasmin is thought to induce TGF- $\beta_1$  activation by directly cleaving LAP from the mature TGF- $\beta_1$  or by releasing active TGF- $\beta_1$  bound to the extracellular matrix (Sato and Rifkin, 1989). Similarly, the matrix-metalloproteinases 2 and 9 (MMP-2 and MMP-9) are thought to proteolytically cleave and activate TGF- $\beta_1$  (Yu and Stamenkovic, 2000). The integrin  $\alpha v \beta 6$ , an epithelial-cell membrane protein, can activate TGF- $\beta_1$  by binding to the RGD sequence in LAP resulting in a conformational change in LAP (Munger et al, 1999). Consistent with a role for this mechanism *in vivo*, animals deficient in the  $\alpha v \beta 6$  integrin develop exaggerated inflammation of the skin and lungs (Huang et al, 1998). However, as expression of the  $\alpha v \beta 6$  integrin is restricted to epithelial cells, this mechanism of activation appears to be specific to the epithelium. Interestingly, the integrin  $\alpha v \beta 8$  can present latent TGF- $\beta_1$  to the transmembrane protease MMP-14 which causes the release of free TGF- $\beta_1$  (Mu et al, 2002). Current evidence suggests that activation of TGF- $\beta_1$  by the matrix protein thrombospondin-1

(TSP-1) is a major activation mechanisms of TGF- $\beta_1$  during post-natal development (Crawford et al, 1998). Mice deficient in TSP-1 show similarities in tissue pathology to TGF- $\beta_1$  knock out mice and this phenotype can be reversed by treatment with a synthetic TSP-1 peptide that activates TGF- $\beta_1$  (Crawford et al, 1998). This thesis has examined the activation of TGF- $\beta_1$  by TSP-1 in fibroblasts and this mechanism will thus be discussed here in more detail.

### **1.6.3. Activation of TGF- $\beta_1$ by TSP-1**

TSPs are a family of five disulphide linked homotrimeric extracellular matrix proteins of which TSP-1 is the most widely characterised isoform (reviewed in (Murphy-Ullrich and Poczatek, 2000)). TSP-1 exists both in a secreted, soluble and also in an insoluble matrix bound state and is expressed by a variety of cells including platelets, endothelial cells and mesangial cells (Reed et al, 1995;Hugo et al, 1995). TSP-1 is involved in platelet aggregation, angiogenesis, cell proliferation, cell adhesion and migration and as previously mentioned it induces the activation of latent TGF- $\beta_1$  (reviewed in (Murphy-Ullrich and Poczatek, 2000)). TSP-1 bound to the cell matrix supports weak cell attachment whereas soluble TSP-1 has anti-adhesive properties and promotes cytoskeletal reorganisation by regulating focal adhesions (Murphy-Ullrich and Hook, 1989). TSP-1 expression *in vitro* can be induced by TGF- $\beta_1$  itself as well as PDGF and fibroblast growth factor-2 (FGF-2) and is particularly found at sites of inflammation and wound healing *in vivo* (Bornstein, 1995). In addition, during the course of this PhD a study revealed that thrombin induces the expression of TSP-1 in endothelial cells (McLaughlin et al, 2005a).

TSP-1 was first shown to be an important activator of TGF- $\beta_1$  by Murphy-Ullrich et al who observed that the inhibitory effects of TSP-1 on platelet growth were partially TGF- $\beta_1$  dependent (Murphy-Ullrich and Poczatek, 2000). Subsequently, a role for TSP-1 mediated activation of TGF- $\beta_1$  was confirmed in both cell culture and cell free systems (Schultz-Cherry et al, 1994). TSP-1 activates TGF- $\beta_1$  by interacting both with the LAP and with TGF- $\beta_1$  itself, thus forming a trimolecular complex, assembly of which is not dependent on the interaction with LTBP (**Figure 1.5.**). The interaction of TSP-1 with TGF- $\beta_1$  requires the binding of the hexapeptide WxxW (WSHW, WSPW or WGPW) from the type 1 repeat of TSP-1 to the active TGF- $\beta_1$  domain (Tsukazaki et al, 1998;Schultz-Cherry et al, 1995).





**Figure 1.5. Activation of TGF- $\beta_1$  by TSP-1**

TSP-1 activates TGF- $\beta_1$  by directly binding to the LAP and TGF- $\beta_1$  forming an active ternary LAP-TSP-1- TGF- $\beta_1$  complex. This interaction requires the binding of the WxxW (WSHW, WSPW or WGPW) from the type 1 repeats of TSP-1 to the active TGF- $\beta_1$  domain, enabling the binding of the second TSP-1 motif (KRFK) to the N-terminal sequence of the LAP. This interaction induces a conformational change in the LAP in such a way that LAP is unable to confer latency to TGF- $\beta_1$  resulting in the generation of active TGF- $\beta_1$  (Figure adapted from (Hugo, 2003)).



This enables the interaction of a second TSP-1 motif, the KRFLK amino acid sequence with the N-terminal LSKL sequence of the LAP (Schultz-Cherry et al, 1994;Schultz-Cherry et al, 1995)), a region of LAP which is implicated in conferring latency to TGF- $\beta_1$  (Sha et al, 1991). Interaction of TSP-1 with the LSKL region is thought to induce a conformational change in the LAP thus leading to the generation of active TGF- $\beta_1$  that is still assembled in the ternary complex (Schultz-Cherry and Murphy-Ullrich, 1993).

The TSP-1-TGF- $\beta_1$  mechanism may be particularly relevant in the activation of TGF- $\beta_1$  in the context of the lung. Thus, TSP-1 null mice develop spontaneous lung inflammation which closely resembles that of mice deficient in TGF- $\beta_1$  (Crawford et al, 1998). Furthermore, *in vivo* TSP-1 inhibition in wild type pups, induced by the intraperitoneal administration of an LSKL peptide, which specifically blocks activation of TGF- $\beta_1$  by TSP-1, has been shown to induce similar pathology (Lawler et al, 1998). Similarly, TSP-1 null mice are protected from spontaneous lung pathology when treated with the TGF- $\beta_1$  activating peptide KRFLK (Lawler et al, 1998). Recent evidence suggests that this mechanism may play a role in the bleomycin model of lung injury and fibrosis (Khalil et al, 1989). Activation of macrophage-derived TGF- $\beta_1$  is mediated by the interaction of plasmin with TSP-1 and its receptor CD36 *in vitro*. Treatment of mice with a synthetic CD36 peptide blocked activation of TGF-  $\beta_1$  and subsequent fibrosis following bleomycin injury (Yehualaeshet et al, 1999).

#### **1.6.4. TGF- $\beta_1$ and fibrosis**

TGF- $\beta_1$  is generally held as the most important mediator of pulmonary fibrosis both in experimental animal models (Khalil et al, 1989) and in human fibrotic lung disease (Khalil et al, 1996). TGF- $\beta_1$  levels as well as most of the TGF- $\beta_1$  -inducible genes (Kaminski et al, 2000) are elevated in the lungs of animals with experimentally-induced lung fibrosis (Coker et al, 1997). Overexpression of TGF- $\beta_1$  leads to the development of progressive fibrosis, whilst inhibition this cytokine using either blocking antibodies or inhibitors targeting TGF- $\beta_1$  signalling pathways affords protection in bleomycin-induced lung injury and fibrosis (Giri et al, 1993). Similarly, TGF- $\beta_1$  levels are increased in BALF of patients with fibrotic lung disease (Ludwicka et al, 1995).

TGF- $\beta_1$  is an important mediator of fibroblast to myofibroblast differentiation via the transcriptional upregulation of  $\alpha$ -SMA and SMAD dependent signalling (Cogan et al, 2002;Subramanian et al, 2004;Chambers et al, 2003). Recent evidence suggests that TGF- $\beta_1$  also plays a role in the differentiation of myofibroblasts from alveolar epithelial cells (Willis et al, 2005). One of the mechanisms implicated in the transcriptional

upregulation of  $\alpha$ -SMA by TGF- $\beta_1$  in fibroblasts is regulation of the cold shock domain protein YB-1, which is a known repressor of  $\alpha$ -SMA transcription (Zhang et al, 2005).

TGF- $\beta_1$  also induces myofibroblast differentiation in granulation tissue *in vivo* (Desmouliere et al, 1993). Administration of TGF- $\beta_1$  to animals before dermal wounding enhances subsequent healing, whilst administration of neutralising antibodies to TGF- $\beta_1$  results in a reduced inflammatory response and delayed wound healing. Importantly, overexpression of TGF- $\beta_1$  in rat lungs results in the development of progressive interstitial and pleural fibrosis which is preceded by the appearance of myofibroblasts (Sime et al, 1997). The ED-A domain (EIIIA) of fibronectin has been implicated as a crucial candidate for the induction of  $\alpha$ -SMA expression by TGF- $\beta_1$ . Tissue injury gives rise to this form of fibronectin and is necessary for TGF- $\beta_1$  to trigger  $\alpha$ -SMA expression and collagen secretion by myofibroblasts although TGF- $\beta_1$  itself has been shown to induce the expression of ED-A fibronectin both *in vivo* and *in vitro* (Serini et al, 1998).

Following wound repair and tissue regeneration myofibroblasts are thought to disappear from sites of injury via the process of apoptosis (Darby et al, 1990). Due to its self limiting nature the disappearance of myofibroblasts also occurs in the bleomycin model of lung injury and fibrosis however in IPF the persistence of the myofibroblast phenotype is thought to contribute to the progressiveness of this disease (Zhang et al, 1994c). TGF- $\beta_1$  is thought to play a major role in inhibiting this process. *In vitro* studies have shown that TGF- $\beta_1$  stimulation may prolong myofibroblast survival (Zhang and Phan, 1999). This may also be a potential mechanism *in vivo* as myofibroblast disappearance in bleomycin-induced lung injury and fibrosis correlates with the decline of TGF- $\beta_1$  (Zhang et al, 1995). One of the cytokines that has been shown to induce myofibroblast apoptosis *in vitro* is IL-1 $\beta$ , which is a known inducer of nitric oxide (NO) (Zhang et al, 1997). This led to the suggestion that NO may be mediating myofibroblast apoptosis *in vivo* (Zhang et al, 1997). TGF- $\beta_1$  has been shown to inhibit this process by at least two mechanisms; the suppression of inducible NO synthase and the prevention of a decline in B cell leukaemia 2 (Bcl-2), which is involved in inhibiting apoptosis (Zhang et al, 1997).

TGF- $\beta_1$  is also the most potent inducer of collagen characterized to date and influences its deposition by a number of mechanisms important in collagen synthesis and degradation. For example, TGF- $\beta_1$  induces procollagen gene transcription (Raghow et al, 1987; Ignatz et al, 1987), increase mRNA stability (Raghow et al, 1987), decreases intracellular degradation of procollagen (McAnulty et al, 1991) and decreases

extracellular degradation of collagen by inhibiting MMP and promoting TIMP production (Overall et al, 1989; Edwards et al, 1987).

Finally, TGF- $\beta_1$  is able to induce the expression of other pro-fibrotic factors in fibroblasts, most notably CTGF (Igarashi et al, 1993).

### **1.7. The role of the coagulation cascade in fibrosis**

Compelling evidence has arisen suggesting that activation of the coagulation cascade with the resultant activation of coagulation proteinases may also play an important role in pulmonary fibrosis (reviewed in (Chambers, 2003)). A recent, non-blinded, randomized small trial in IPF of prednisolone alone or prednisolone plus anticoagulation (oral warfarin for outpatients or low molecular weight heparin for hospitalized patients) (Kubo et al, 2005) reported a significant increase in survival in the anticoagulant group, by reducing mortality associated with acute exacerbation. Despite some methodological caveats, this remains one of the most significant beneficial outcomes on survival in an IPF clinical study to date. This thesis has specifically focused on the role of the coagulation cascade in pulmonary fibrosis and this section will therefore describe the coagulation cascade in more detail.

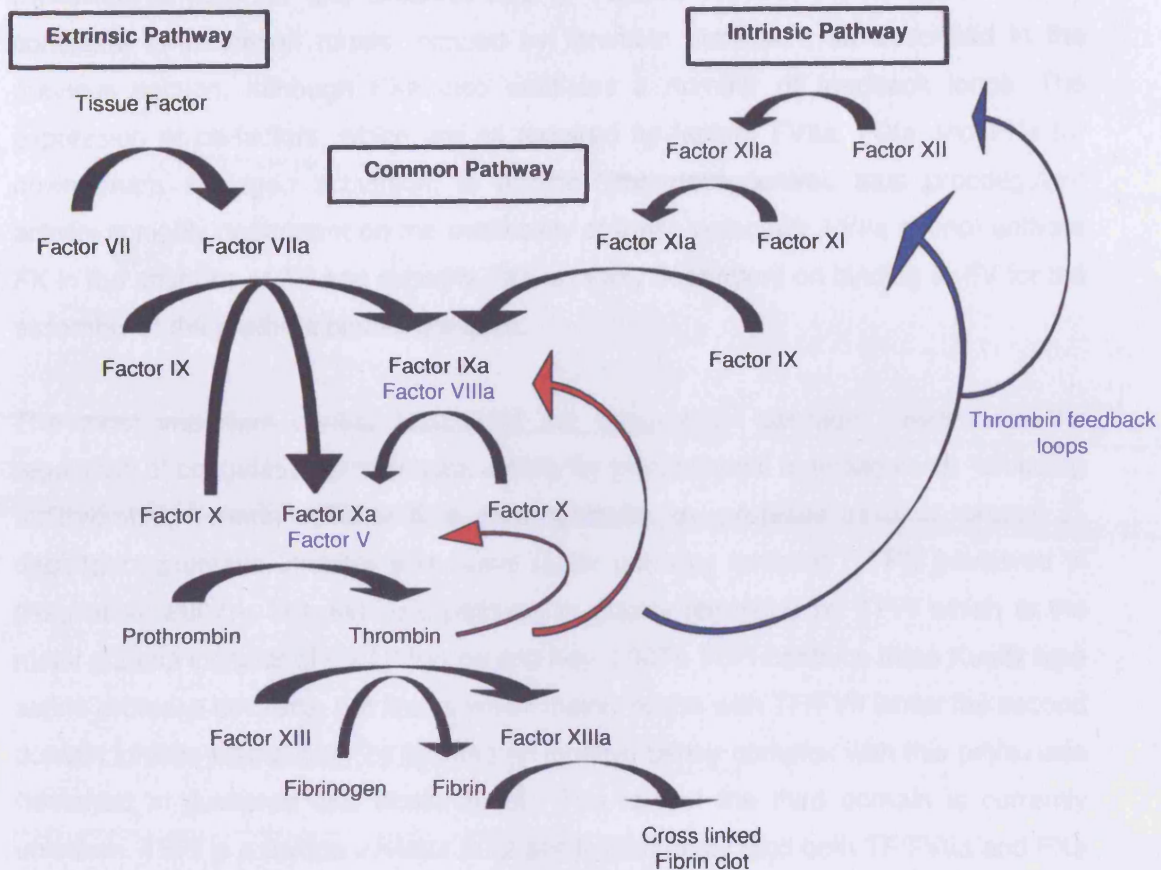
#### **1.7.1. The coagulation cascade**

One of the earliest events following tissue injury is the activation of the coagulation cascade. The primary role of the coagulation cascades is to ensure the formation of stable haemostatic clots to prevent blood loss in response to damage to the extensive lung capillary and microcapillary vasculature. Following tissue injury, successive steps of coagulation proteinase activation via the intrinsic and extrinsic pathways of the coagulation cascade result in the generation of thrombin and fibrin (reviewed in (Mann et al, 2003a)). This process will be reviewed in the following section and a diagram representing the coagulation cascade is represented below (**Figure 1.6.**).

The activation of the coagulation cascade and the eventual formation of fibrin is a highly regulated process (reviewed in (Mann et al, 2003b)). The events occur most efficiently when restricted to a phospholipid membrane found on activated platelets, damaged extravascular cells and procoagulant phospholipids (microparticles) which allows high concentration of the proteinases to accumulate locally and thus increase enzymatic reactions by several orders of magnitude. In addition, non-lipid components expressed on cell surfaces such as protein binding sites may be influencing the

generating capacity of active proteinases (Monroe et al, 2002). These processes are highly dependent on the presence of calcium ions, which enable proteinase binding to phospholipid membranes and further enhance proteinase activation. Further regulation by circulating and endogenous anticoagulants ensures that clot formation is localised to the site of initial injury and thus prevents disregulated activation at non-involved sites (please see also **Section 1.7.2.**).

Coagulation proteinases circulate in the blood as inactive zymogens and are activated by limited proteolysis (reviewed in (Riewald and Ruf, 2002)). The major activator of the coagulation cascade *in vivo* is the extrinsic pathway. It is initiated when injury to the vessel wall causes blood components to come into contact with the glycoprotein receptor tissue factor (TF). This receptor is usually expressed at very low levels and exclusively on extravascular cells such as smooth muscle cells, fibroblasts and endothelial cells, as well as platelets (Mann et al, 2003a). This ensures that blood components are physically separated from TF under normal circumstances. At sites of vascular endothelial damage, upregulation of TF expression on extravascular cells allows blood components such as FVII to come into contact with TF which subsequently activates and thus converts FVII to FVIIa. The resulting vitamin K-dependent TF/FVIIa complex binds to FX to form the so called extrinsic tenase complex (TF/FVIIa/FX) which catalyses the conversion of FX to FXa and also induces the conversion of limited amounts FIX to FIXa. The resulting ternary complex (TF/FVIIa/FXa) is thought to be transient as a small amount of FXa dissociates and forms the prothrombinase complex (FVa, FXa, phospholipids, calcium) which converts prothrombin to thrombin. The activation of thrombin is a two stage process, involving the formation of an intermediate thrombin molecule, termed meizothrombin, which however stays bound to the complex in a proteolytically active form until secondary cleavage produces the mature thrombin molecule. Thrombin in turn converts fibrinogen to fibrin, activates FV, FVIII, FXI and FXII and platelets which cross link the provisional fibrin clot and thus form a stable haemostatic plug. However, the generation of thrombin via the extrinsic coagulation pathway is thought to only generate limited amounts of thrombin. Continuous activation of the coagulation cascade is achieved by the activation of factors FXI, FIX, FVIII by the TF/FVIIa/FXa ternary complex. This initiates the activation of the intrinsic pathway which plays an important role in mediating sustained generation of thrombin. Thus following vascular injury initial activation of the extrinsic pathway generates trace amount of thrombin and FXa. These feed back into the intrinsic pathway, which serves as an amplification step leading to the continuous activation of thrombin and thus sustained coagulation activity.



**Figure 1.6. The coagulation cascade.**

The catalytic activity of most coagulation proteinases is regulated by their binding to cell surface phospholipids and the presence of calcium ions ( $\text{Ca}^{2+}$ ). In addition, FIXa and FXa only gain their full activity when complexed with their respective cofactors, FVIIIa and FVa. Finally, thrombin activates the intrinsic pathway via positive feedback mechanisms. Please note that only the procoagulant events and positive feedback mechanisms are illustrated for clarity.

### 1.7.2. Regulation of the coagulation cascade

The coagulation cascade is tightly regulated by a number of positive and negative feedback mechanisms, co-factor expression as well as endogenous anticoagulants (reviewed in (Laurent and Shapiro, 2007)). Positive feedback mechanisms mainly constitute amplification routes initiated by thrombin activation, as described in the previous section, although FXa also mediates a number of feedback loops. The expression of co-factors, which are all required by factors FVIIa, FIXa and FXa for downstream zymogen activation, is another important control; thus procoagulant activity is highly dependent on the availability of these cofactors. FVIIa cannot activate FX in the absence of TF and similarly FXa is highly dependent on binding to FV for the assembly of the prothrombinase complex.

The most important control feature of the coagulation cascade however, is the regulation of coagulation proteinases activity by physiological anticoagulants including antithrombin, heparin cofactor II,  $\alpha_2$ -macroglobulin,  $\alpha_1$ -protease inhibitor, protein Z-dependent protease inhibitor and tissue factor pathway inhibitor (TFPI) (reviewed in (Rau et al, 2007)). The extrinsic pathway is mainly regulated by TFPI which is the major plasma inhibitor of FVII (Monroe and Key, 2007). TFPI contains three Kunitz type serine protease domains, the first of which mainly reacts with TF/FVII whilst the second domain inhibits FXa activity by forming an inactive binary complex with this proteinase (reviewed in (Lwaleed and Bass, 2006)). The role of the third domain is currently unknown. TFPI is a unique inhibitor in its ability to strongly bind both TF/FVIIa and FXa to form a quaternary complex, but leaving low levels of FVIIa active. This is due to its inability to bind free FVIIa which results in a highly sophisticated inhibitory mechanism that allows the coagulation cascade to be triggered when necessary but induces the rapid shut-down of the initiation complex, preventing any ongoing activation. TFPI is by far the most potent inhibitor of the coagulation cascade, and mice deficient in the first Kunitz domain die *in utero*, suggesting that a deficiency in this inhibitor is not compatible with life (Huang et al, 1997).

The main endogenous inhibitor of the intrinsic pathway is antithrombin, which is a single-chain glycoprotein that belongs to the serpin family and that is not dependent on the presence of vitamin K. Antithrombin irreversibly neutralises thrombin and also inhibits FXIIa, FXIa, FIXa and FXa (reviewed in (Rau et al, 2007)). The presence of heparin is required for these processes to occur efficiently, although extremely high levels of heparin are needed for antithrombin-mediated inhibition of FVIIa thus questioning the physiological relevance of this process. Deficiency in antithrombin

leads to widespread venous thrombosis indicating the importance of this inhibitor in regulating coagulation activity. In addition to their inhibitory role during blood coagulation, antithrombin and TFPI also play a role in the catabolism of the inhibited proteinase. Within minutes of their inhibition, the antiproteinase-proteinase complexes are cleared from the blood and targeted to the liver where they are finally degraded (Narita et al, 1998;Ho et al, 1996).

Finally, the coagulation cascade is controlled when low concentrations of thrombin bind to thrombomodulin, an integral membrane protein expressed on the surface of endothelial cells (reviewed in (Mann et al, 2003b)). Binding of thrombin to thrombomodulin induces a conformational change in the proteinase thereby changing thrombin's substrate specificity and essentially converting it from a procoagulant into an anticoagulant factor. In contrast to its role during coagulation, thrombin in this conformation preferentially cleaves the inactive zymogen protein C thus converting it to its active form, activated protein C (APC). This reaction is highly dependent on the zymogen protein C binding to the endothelial protein C receptor (EPCR) and activated protein S which acts as its cofactor. APC controls coagulation by influencing the activity of the FIXa/FVIIIa and FXa/FVa complexes through proteolytic inactivation of their cofactors FVa and FVIIIa. This mechanism also prevents further feedback generation of thrombin as these factors are then unable to form the prothrombinase and intrinsic tenase complexes.

### ***1.7.3. Evidence for the role of the coagulation cascade in pulmonary fibrosis***

It is increasingly recognised that the coagulation cascade is activated in fibrotic lung disease and may contribute to the pathophysiology of pulmonary fibrosis. Extravascular intra-alveolar accumulation of fibrin has been described for patients with pulmonary fibrosis (Chapman et al, 1986; Ikeda et al, 1989; Kotani et al, 1995), organising pneumonia (Peyrol et al, 1990), acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) (Bachofen and Weibel, 1982; Idell et al, 1987; Idell et al, 1989). Extensive research into the role of the coagulation cascade in the past years has emphasised the importance of the TF-dependent extrinsic pathway in the extravascular space and it is now generally held that this pathway is activated in fibrotic lung disease. Evidence for this has been provided by studies reporting strong immunoreactivity for TF, the main initiator of the extrinsic pathway, on type II pneumocytes as well as on macrophages that are closely associated with fibrin deposits in patients with idiopathic pulmonary fibrosis, as well as with systemic sclerosis (Imokawa et al, 1997). Increased TF expression can be mediated by TNF- $\alpha$

and TGF- $\beta_1$  stimulation in cultured epithelial cells (Idell et al, 1994). In addition, TF expression is also upregulated in epithelial cells, macrophages and fibroblasts in the bleomycin model of lung injury and fibrosis (Wygrecka et al, 2007b; Idell, 2003).

The persistent activation of the coagulation cascade is thought to arise from an imbalance between pro- and anti-coagulant factors. For example, increased levels of the coagulation proteinase thrombin have been measured in the BALF of patients with pulmonary fibrosis associated with systemic sclerosis (Hernandez Rodriguez et al, 1995; Ohba et al, 1994), in pulmonary fibrosis associated with chronic lung disease of prematurity (Dik et al, 2003b) and in experimental animal models of pulmonary fibrosis such as those induced by radiation-pneumonitis (Huang et al, 2001) or by administration of bleomycin (Tani et al, 1991; Howell et al, 2001). Increased levels of other coagulation proteinases including, fibrinogen and Factors FVII and FX have also been described in patients with intra-alveolar fibrosis associated with BOOP/COP (Peyrol et al, 1990). Similarly, levels of endogenous anticoagulants such as antithrombin in patients with ARDS (a condition associated with fibroproliferative changes in 40% of cases, reviewed in (Ware et al, 2006)) and lower levels of APC have been measured in patients with interstitial lung diseases respectively (Yasui et al, 2000).

#### **1.7.4. Fibrinolysis in the fibrotic lung**

Current evidence suggests that fibrinolysis, which is involved in the clearance of deposited fibrin, is dysregulated in fibrotic lung disease (Swaisgood et al, 2000; Bertozzi et al, 1990). The process of fibrinolysis is initiated by the proteolytic conversion of plasminogen leading to the generation of plasmin by the proteinases, urokinase type plasminogen activator (u-PA) and tissue type plasminogen activator (t-PA) (reviewed in (Kucharewicz et al, 2003)). The major role of plasmin is the cleavage of fibrin into several fibrin degradation products (FDP). t-PA is the main converter of plasminogen in plasma, whilst u-PA controls the fibrinolytic system primarily in the extravascular space. Similarly to the coagulation system, the activity of the fibrinolytic system is regulated by several endogenous inhibitors, including the plasma glycoprotein, thrombin-activatable fibrinolysis inhibitor (TAFI), protein C inhibitor (PCI) and plasminogen activator inhibitor-1 (PAI-1). TAFI promotes fibrin persistence by cleaving specific C-terminal lysine residues which are exposed on fibrin molecules during fibrinolysis. PAI-1 is the main regulator of plasminogen cleavage by either u-PA or t-PA and PCI suppresses plasminogen activation and further inhibits the activity of APC. Increased levels of all these inhibitors have been reported in patients with ILDs and in experimental animal



models of pulmonary fibrosis (Fujimoto et al, 2003). The resulting decrease in alveolar fibrinolytic activity thus leads to reduced degradation of extracellular matrix and promotes fibrin persistence in the fibrotic lung (Swaisgood et al, 2000).

#### **1.7.5. Targeting the coagulation cascade**

A number of studies have provided compelling evidence that targeting the coagulation cascade is protective in experimental animal models of pulmonary fibrosis. For example, previous work in our laboratory has shown that direct thrombin inhibition leads to a significant reduction in lung collagen accumulation in the bleomycin model of lung injury and fibrosis in rats (Howell et al, 2001). Similarly, intratracheal instillation of the endogenous anticoagulant APC is protective in mice following bleomycin injury (Yasui et al, 2001) although it is currently unclear from this study whether APC exerts its inhibitory effects via its anticoagulant or anti-inflammatory properties. Administration of nebulised heparin in rabbits, which binds to antithrombin and increases its ability to inhibit thrombin, FIXa, and FXa, has also been shown to reduce bleomycin-induced lung injury and fibrosis (Gunther et al, 2003). Finally, intratracheal gene transfer of TFPI has been shown to exert profound protective effects on bleomycin-induced lung injury and fibrosis in mice by reducing both thrombin activity, as well as lung collagen accumulation (Kijiyama et al, 2006).

#### **1.7.6. Mechanisms by which the coagulation cascade exerts its pro-fibrotic effects**

The role of fibrin deposition in the pathogenesis of pulmonary fibrosis has recently been questioned and remains controversial (Hattori et al, 2000). Fibrin is thought to influence fibrogenesis in a number of ways. First excessive deposition of fibrin is thought to adversely influence alveolar surfactant function thus leading to alveolar collapse or atelectasis. Second, *in vitro* studies have demonstrated that fibrinogen can induce the expression and release of a host of pro-inflammatory mediators from neutrophils (Walzog et al, 1999), macrophages (Smiley et al, 2001), endothelial cells (Qi et al, 1997) and fibroblasts (Liu and Piela-Smith, 2000) which may be directly influencing fibroblast recruitment and differentiation and thus collagen deposition. Third, fibrin serves as a reservoir of fibrogenic growth factors and cytokines which are released during fibrinolysis (Grainger et al, 1995). Studies directly altering the activity of the fibrinolytic system by either promoting or inhibiting its activity support a role of fibrin in the pathogenesis of pulmonary fibrosis (Eitzman et al, 1996; Gunther et al, 2003; Fujimoto et al, 2003). However, a recent study reporting the finding that fibrinogen knock-out mice are not protected from bleomycin-induced lung injury and

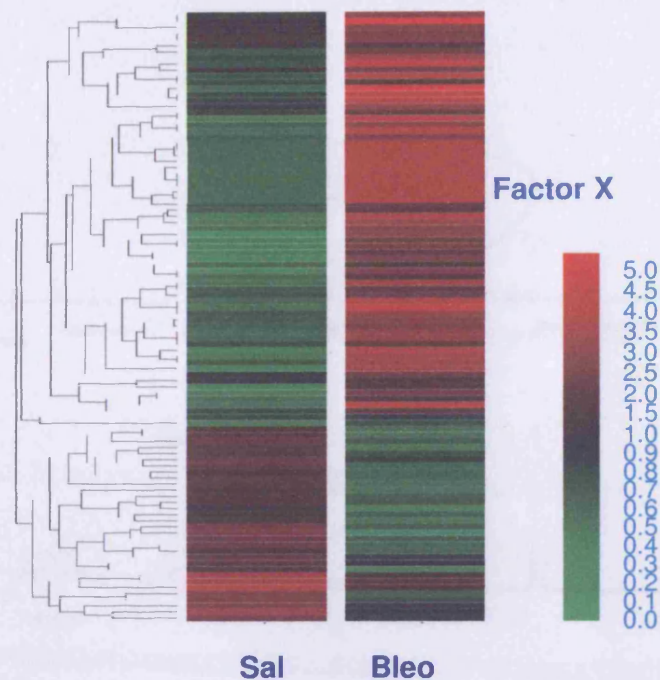
fibrosis has suggested that fibrin deposition is necessary but not sufficient for the progression of fibrosis, at least in this model (Hattori et al, 2000).

There is increasing evidence that the coagulation cascade may promote fibrogenesis through the ability of coagulation proteinases of the extrinsic pathway to directly induce cellular effects which may be important in influencing inflammatory and tissue repair responses (Chambers, 2003; Kijiyama et al, 2006). In particular, the pro-fibrotic effects of thrombin have received considerable attention, although recent work has suggested that coagulation proteinases upstream of thrombin in the coagulation cascade, including FXa may also play an important role. The work presented in this thesis has specifically focussed on the role of FXa. Despite the traditional view that FXa is exclusively synthesised in the liver (Hung and High, 1996), a recent exciting transcriptional profiling study performed in our laboratory revealed that FX was expressed in the lung (**Figure 1.7.**). As part of a wider microarray global expression study Drs Scotton and Lee compared gene expression profiles of mice treated with saline or bleomycin and identified numerous genes that were differentially regulated between the groups. Amongst the most highly regulated genes was the zymogen FX. This led to the hypothesis that FX is synthesised locally in the lung and may play a role in the bleomycin model of lung injury and fibrosis. This hypothesis forms a major part of this PhD work and the next section will thus discuss the structure and synthesis of this proteinase in detail.

## 1.8 Coagulation Factor X

### 1.8.1. Structure

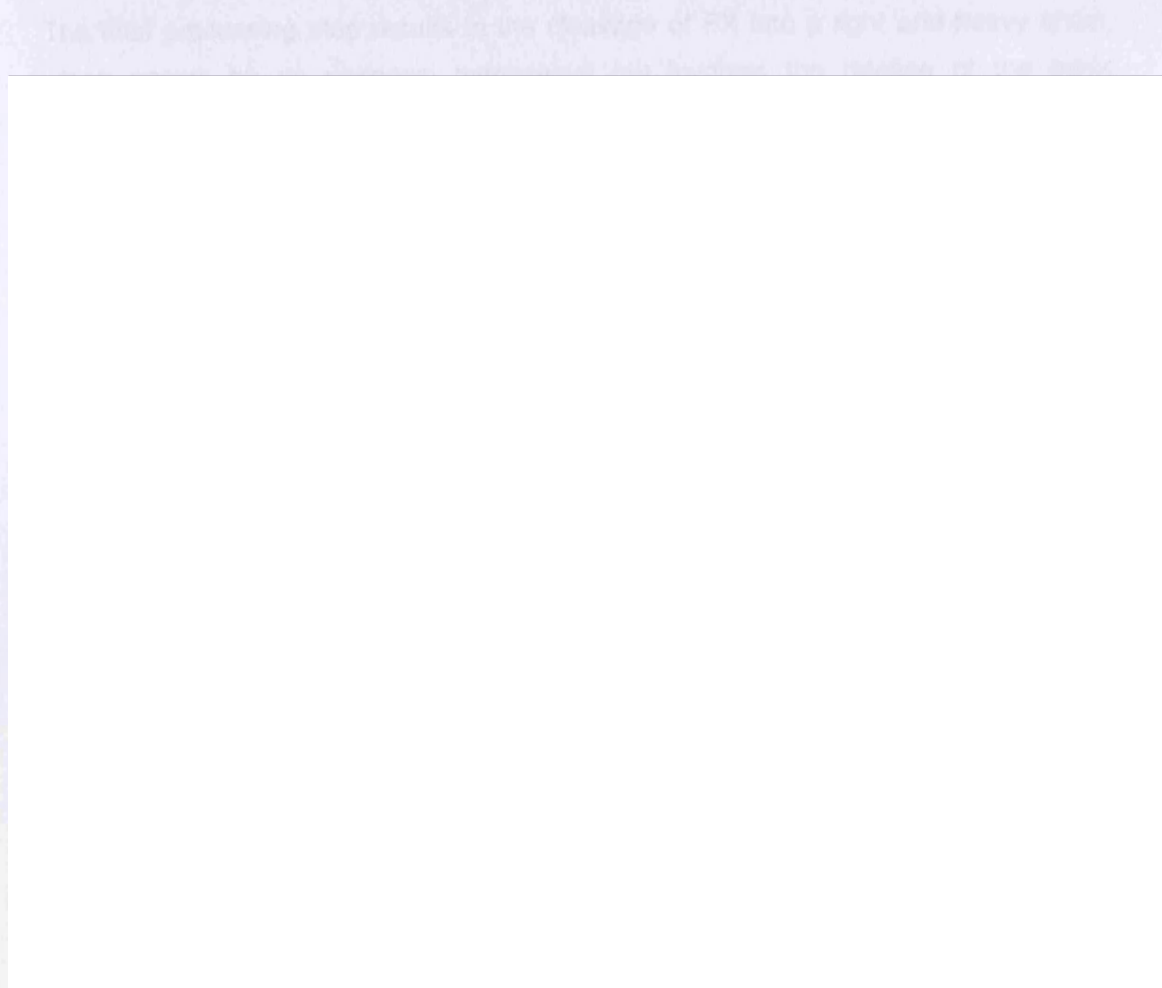
FX is a glycoprotein that is synthesised in the liver and circulates in plasma as a precursor to the active coagulation proteinase FXa (reviewed in (Laurent and Shapiro, 2007)). The gene for FX is located in chromosome 13 at position q32-qter. It is 27 kb long and contains eight exons and seven introns, resulting in the transcriptional generation of a 1.5 kb mRNA molecule. 1475 nucleotides of the mRNA code for the pre-pro-leader sequence of 40 amino acids, a light chain of 139 amino acids and a connecting peptide of 303 amino acids that makes up the heavy chain. The protein is synthesised in the liver by hepatocytes, as a single-chain 70 kDa precursor, the pre-proprotein, of 488 amino acids with 24 half cystines. This FX precursor undergoes a number of posttranslational modification steps before it circulates in the plasma as the mature two chain zymogen (**Figure 1.8.**).



**Figure 1.7. Transcriptional profiling of bleomycin-induced lung injury and fibrosis**

Heat map showing hierarchical clustering of the top 100 genes differentially regulated 14 days following bleomycin instillation in mice. Colour represents mRNA expression levels from low (green) to high (red). FX was one of the most highly upregulated genes (data provided by Dr Chris Scotton, UCL).

These include translocation of the newly formed precursor protein to the endoplasmic reticulum where the pre-peptide signal is cleaved off by a signal peptidase (Wallin and Turner, 1990). The remaining pro-peptide directs the subsequent  $\gamma$ -carboxylation by the vitamin K dependant  $\gamma$ -carboxylase which converts 10-12 glutamic acid residues in the N-terminal part of the protein into  $\gamma$ -carboxyglutamic acid (Gla) residues, an essential modification to ensure optimal activation of the zymogen once secreted (Furie and Furie, 1988). Following cleavage of the propeptide by a processing protease, the FX precursor undergoes  $\beta$ -hydroxylation of Asp63 in the first EGF-like domain and a high degree of N-linked glycosylation close to the side of the activation peptide region, which increases the relative mass of FX from 58.2 kDa to 58.9 kDa.



**Figure 1.8. Processing and activation of FX**

**(A)** FX polypeptide before processing. FX protein is synthesised in the liver by hepatocytes as a single-chain 70kDa precursor, containing the pre-propeptides,  $\gamma$ -carboxyglutamic acid (Gla) domain, EGF-like repeats, activation peptide and protease domain.  $\gamma$ = Gla residues,  $\beta$ = $\beta$ -hydroxylated Asp<sub>63</sub> **(B)** Processed FX protein. During post-translational modification, the prepeptide signal sequence is cleaved off by a signal peptidase while the propeptide directs  $\gamma$ -carboxylation of the first 11 Glu residues of the N-terminus (Gla domain). After further intracellular processing, FX is secreted into the blood as a glycosylated, two-chain, disulphide-linked inactive zymogen. **(C)** Activated FX, FXa. FX is activated via hydrolysis of a peptide bond at a single site (Arg<sub>194</sub>) in the heavy chain to release a small 52 amino acid activation peptide, catalyzed by either TF/FVIIa or FIXa/FVIIa (Image taken from (Laurent and Shapiro, 2007)).

The final processing step results in the cleavage of FX into a light and heavy chain, which occurs by an unknown mechanism but involves the release of the basic tripeptide Arg140-Lys-Arg142 from the centre of the molecule. The 16.2 kDa light chain contains the Gla and EGF-like domains whilst the activation peptide and the latent serine protease domain are situated on the 42 kDa heavy chain. The two chains are additionally linked together by a disulphide bond between Cys<sub>132</sub> and Cys<sub>302</sub>. It is these two chains that make up the mature form of FX and both are required for FX to exhibit its procoagulant and cellular effects once activated. Although this structure shares high homology to the trypsin family, FXa confers specificity for its substrates and inhibitors through the insertion of various sized peptide sequences, called insertion loops onto the external surface of the molecule.

### 1.8.2. FX activation

FX is activated by hydrolysis of a peptide bond at a single site (Arg<sub>194</sub>) from the N-terminus of the heavy chain to release a small 52 amino acid activation peptide (Mr=8000), providing a potentially measurable marker of FXa activation ((Di Scipio et al, 1977), **Diagram 1.8.**). As already eluded to in previous sections FX, cleavage can be catalysed by either FIXa/FVIIIa complex or by the TF/FVIIa complex and both reactions generate the identical FXa product. Activation of FX by the FIXa/FVIIIa complex plays a central role in the intravascular space as FIXa-deficient mice are completely protected from thrombus formation in vascular injury models (Gui et al, 2007). In contrast, it is generally believed that the TF/FVIIa complex plays a major role in the activation of FX in environments of high TF expression, such as those generated in the extravascular space post tissue injury (Chambers and Laurent, 2002). However, FX can be activated by alternative mechanisms independently of either intrinsic or extrinsic coagulation pathway activity. Thus in inflammatory environments, FX may bind to CD11b/CD18 (MAC-1) expressing monocytic cells, which subsequently mediate FX cleavage via the secretion of the proteinase cathepsin G (Plescia and Altieri, 1996). FXa activation may also be mediated by invading organisms during infectious states. Viruses such as cytomegalovirus, herpes simplex virus types 1 and 2 all display the appropriate phospholipids to promote FXa generation in a FVII-dependent but TF-independent manner (Sutherland et al, 1997). In addition, certain bacterial proteinases, such as gingipain-Rs from *Porphyromonas gingivalis*, can cleave and directly activate FX and thereby initiate the activation of the coagulation cascade (Imamura et al, 1997).

### **1.8.3 Biological functions of FXa**

FXa exerts several cellular effects which may directly influence inflammatory responses. For example, FXa induces the secretion of various pro-inflammatory cytokines, including IL-1 by fibroblasts (Jones and Geczy, 1990), IL-2 by lymphocytes (Altieri and Stamnes, 1994) as well as IL-6 and IL-8 by endothelial cells (Senden et al, 1998). Some of these effects appear to be mediated via the initial release of NO, which additionally affects the inflammatory reaction and vascular tone. FXa also induces oedema formation when injected subcutaneously in a rat paw inflammation model, via the local recruitment of mast cells (Cirino et al, 1997). Furthermore, FXa mediates the expression of adhesion molecules on monocytes (Senden et al, 1998) and induces NK- $\kappa$ B and Cyr61 gene expression in HeLa cells (Riewald et al, 2001).

FXa is a potent mitogen for a variety of cell types. In smooth muscle cells, FXa stimulates DNA synthesis and mitogenesis via the autocrine stimulation of PDGF (Herbert et al, 1998; Gasic et al, 1992) or epiregulin (Koo and Kim, 2003). Similarly, FXa stimulates endothelial cell proliferation via autocrine production of PDGF (Gajdusek et al, 1986). FXa is also mitogenic to mesangial cells (Tanaka et al, 2005; Tanaka et al, 2005) and to lymphocytes and the mitogenic effect on lymphocytes is thought to be mediated via the release of IL-2 (Altieri and Stamnes, 1994). FXa is further known to modulate tumour cell growth, invasion and metastasis (Sampson and Kakkar, 2002).

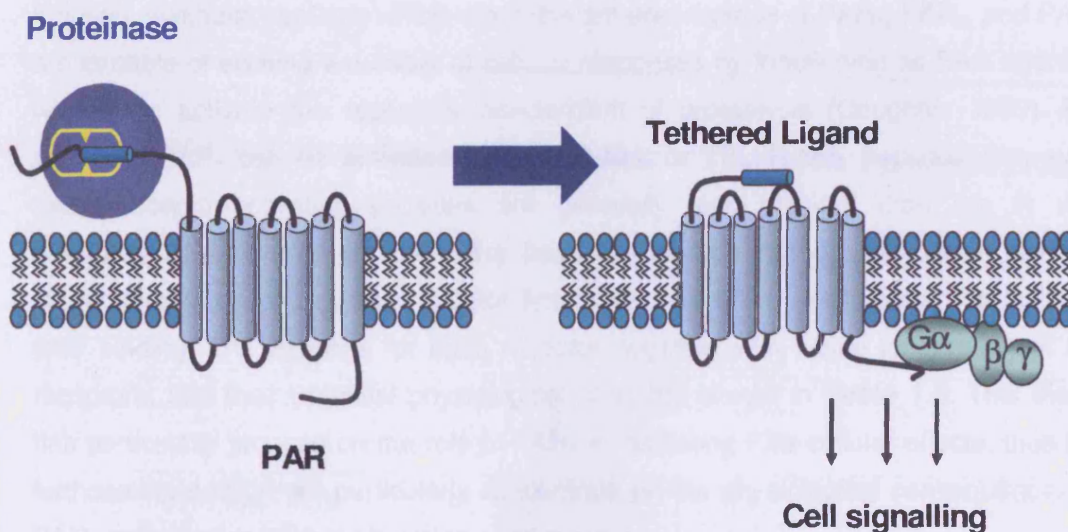
The contribution of FXa's cellular effects to pathophysiological processes has only just begun to unfold. Recent evidence from our laboratory has implicated a role for FXa in stimulating fibroblast responses which may contribute to the pathogenesis of fibrotic lung disease (Blanc-Brude et al, 2005). At the same time, evidence was provided that similar to thrombin, FXa exerted its effects on certain cell types via its ability to activate members of the novel family of seven transmembrane G-protein coupled receptors called proteinase activated receptors (PARs). This evidence will now be discussed in greater detail.

### **1.9. Proteinase Activated Receptors (PARs)**

The quandary as to how coagulation proteinases, such as FXa and thrombin, influence cellular responses was elucidated by the discovery of the PARs in the early 1990s (Ossovskaya and Bunnett, 2004). The PARs are a novel family of ubiquitously expressed seven transmembrane domain G-coupled protein receptors. As their name



suggests, the receptors are activated by a unique mechanism involving limited proteolysis of a specific sequence at the N-terminus by the proteinase, leading to the unmasking of a ligand which is tethered to the receptor. This tethered ligand binds to the second extracellular loop, which induces a conformational shape change in the receptor, allowing it to interact with heterotrimeric G-proteins and thus initiate downstream signalling (**Figure 1.9.**).



**Figure 1.9. Activation of proteinase-activated receptors**

Following proteolytic cleavage of PARs, the newly generated tethered ligand binds to the second extracellular loop of the receptor. This induces a conformational change in the receptor, allowing it to interact with heterotrimeric G-proteins and initiate downstream signalling responses.

Currently, the PAR family comprises four members, PAR<sub>1</sub> to PAR<sub>4</sub>. All four PAR genes share a homologous structure. They contain two exons of which exon 1 encodes a signal peptide and exon 2 the mature receptor protein (Kahn et al, 1998). The genes for PAR<sub>1-3</sub> are all located on the same chromosome locus (5q13), whilst PAR<sub>4</sub> is located on 19p12 (Xu et al, 1998). Collectively, the proteinases of the coagulation cascade can activate all four members of the PAR family. Thrombin is considered the major activator of PAR<sub>1</sub>, PAR<sub>3</sub> and PAR<sub>4</sub>, whereas FXa activates PAR<sub>1</sub> and PAR<sub>2</sub>. However, there is increasing evidence that PARs can also be activated by non-coagulation proteinases. For example, trypsin and tryptase are major activators of PAR<sub>2</sub> and a recent report has suggested that PAR<sub>1</sub> can also be activated by the matrix metalloproteinase, MMP1 (Boire et al, 2005). To dissect the different PARs in terms of function, synthetic peptides which mimic the tethered ligands of PAR<sub>1</sub>, PAR<sub>2</sub>, and PAR<sub>4</sub> are capable of eliciting a number of cellular responses by functioning as PAR agonists which can activate the receptors independent of proteolysis (Coughlin, 1999). For example, PAR<sub>1</sub> can be activated by SFLLR-NH<sub>2</sub> or TFLLR-NH<sub>2</sub> peptides. However, these receptor-activating peptides are generally less efficient than the *in vivo* physiological activators of the PARs because they do not enable certain specific interactions to occur between receptor and proteinase which are important to facilitate their binding. The agonists for each receptor, together with tissue localization of the receptors, and their potential physiological roles are shown in **Table 1.5**. This thesis has particularly focused on the role of PAR<sub>1</sub> in mediating FXa cellular effects, thus the forthcoming section will particularly concentrate on the physiological consequences of PAR<sub>1</sub> activation in normal physiology and disease.

### 1.9.1. PAR<sub>1</sub>

As already alluded to, PAR<sub>1</sub> is the major cellular receptor for thrombin, although it can also be activated by FXa, the ternary complexes (TF/FVIIa /FXa) (Riewald et al, 2001), activated protein C (Riewald et al, 2002), and plasmin (Mandal et al, 2005). Thrombin is a very efficient activator of PAR<sub>1</sub> which is facilitated by a hirudin-like binding domain situated at N-terminus of PAR<sub>1</sub>. The attraction between this domain and thrombin's anion binding exosite enables thrombin to effectively complex with PAR<sub>1</sub> and thus initiate cleavage of the receptor. In contrast, FXa lacks this binding side but efficient activation of PAR<sub>1</sub> by this proteinase is facilitated by FXa complexing with TF and FVIIa at the site of its generation, so that the proteinase is localised to the cell membrane. In this constellation FXa is five times more potent at activating this receptor compared to FXa alone (Riewald et al, 2001). Similarly, APC utilizes the cell surface receptor EPCR as a cofactor to facilitate its activation of PAR<sub>1</sub> (Riewald et al, 2002).



**Table 1.5. Proteinase-activated receptors (PARs)**

*A summary of PAR activating proteases, disabling proteases, activating peptides, localization and phenotypes of PAR deficient mice. Updated from (Ossovskaia and Bunnett, 2004)*

**1.9.2. PAR<sub>1</sub> signalling pathways**

PAR<sub>1</sub> exerts its multiple cellular effects via interaction with downstream G-proteins. G proteins are heterotrimeric guanine nucleotide binding proteins which consist of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. G $\alpha$  is in its inactive form when bound to GDP. Following cleavage of the receptor by the proteinase, a conformational change in the receptor initiates the phosphorylation and thus conversion of GDP to GTP. This induces the dissociation of the  $\alpha$  subunit from the  $\beta\gamma$  subunits and thus initiates cell signalling. Three main classes of G proteins can be distinguished according to the primary sequence of the  $\alpha$  subunit

and PAR<sub>1</sub> is able to couple to G $\alpha_i$ , G $\alpha_q$  and G $\alpha_{12/13}$  (reviewed in (Coughlin, 1999)). The type of G protein to which PAR<sub>1</sub> is coupled to dictates the nature of the cellular response (reviewed in (Hollenberg and Compton, 2002)). In addition, it is increasingly recognized that in the same cell type, responses to different agonists may vary greatly. For example, thrombin and activated protein C (APC) which both signal via PAR<sub>1</sub> in TNF- $\alpha$  perturbed endothelial cells have been reported to mediate distinct biological effects (Riewald and Ruf, 2005). This observation may be explained by the phenomenon of functional selectivity whereby different agonists of PAR<sub>1</sub> selectively activate different G-protein pathways by their ability to alter receptor/G protein binding (McLaughlin et al, 2005b). Thus, thrombin and the PAR agonist peptide, TFLLR, both activate PAR<sub>1</sub> on endothelial cells to influence barrier permeability and calcium mobilization. However activation by thrombin is associated with more efficient receptor coupling to G $\alpha_{12/13}$  and a greater effect on barrier permeability, whereas activation by TFLLR is associated with more efficient receptor coupling to G $\alpha_q$  and a greater effect on calcium mobilization (McLaughlin et al, 2005b).

PAR<sub>1</sub> impacts on a substantial network of signalling pathways, as shown in **Figure 1.10**. The cell signalling pathways involve multiple interactions at a number of levels, and are very cell-specific.

#### *PAR<sub>1</sub>-G $\alpha_i$ pathway*

PAR<sub>1</sub> coupling to various G-protein  $\alpha$  subunits is mediated by the presence of different binding sites on the receptor. For example, the third intracellular loop of PAR<sub>1</sub> is involved in PAR<sub>1</sub> coupling to the pertussis-toxin sensitive G $\alpha_i$ . Coupling to the G $\alpha_i$  subunit leads to downstream inhibition of adenylyl cyclase and reduces the generation of cyclic adenosine monophosphate (cAMP) and potentiates calcium ion mobilisation in response to thrombin-mediated activation (Hung et al, 1992b). PAR<sub>1</sub>- G $\alpha_i$  coupling also activates Src tyrosine kinase, which triggers the Ras, Raf and mitogen-activated protein kinase (MAP kinase) pathway. This pathway is necessary for thrombin-and FXa-induced fibroblast proliferation (Blanc-Brude et al, 2005; Trejo et al, 1996). Recent studies have also shown that PAR<sub>1</sub>- G $\alpha_i$  coupling is responsible for the increased expression of thrombospondin-1 (TSP-1) induced by thrombin in vascular endothelial cells (Riewald and Ruf, 2005). Binding of PAR<sub>1</sub> to G $\alpha_i$  is also necessary for thrombin-induced changes in endothelial barrier permeability (Riewald and Ruf, 2005).



**Figure 1.10. Major PAR<sub>1</sub> G-protein coupled signalling pathways**

PAR<sub>1</sub> exhibits the ability to couple to multiple G-protein family subunits, including G $\alpha_i$ , G $\alpha_q$  or G $\alpha_{12/13}$  within the same cell type. In general, the G $\alpha_i$  pathway inhibits adenylyl cyclase and the generation of cyclic adenosine monophosphate (cAMP); the G $\alpha_q$  pathway involves phospholipase C- $\beta$  (PLC- $\beta$ ) activation and concomitant calcium mobilization and PKC activation; whereas the G $\alpha_{12/13}$  pathway activates Rho kinase and regulates actin remodelling. (Figure modified from (Coughlin, 2000; Ossovska and Bunnett, 2004))

*PAR<sub>1</sub>-Gα<sub>q</sub>-pathway*

A major PAR<sub>1</sub> signalling pathway is through coupling to Gα<sub>q</sub> (Hung et al, 1992a). The c-terminal tail of PAR<sub>1</sub> is required for the interaction with Gα<sub>q</sub>. Gα<sub>q</sub> activates the phospholipase-β (PLC-β) pathway (Babich et al, 1990) which subsequently triggers phosphoinositide hydrolysis (PIP<sub>2</sub>) to generate inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). This in turn mobilises calcium from intracellular stores, increasing the intracellular calcium concentration from approximately 10<sup>-7</sup>M to 10<sup>-3</sup>M and also causes the activation of protein kinase C (PKC) (Berridge, 1993). Calcium, as a second messenger, activates calcium-dependent kinases and phosphatases such as CaM kinases, Ras guanine-nucleotide exchange factors (GEFs) and MAP kinases. PKC is a key enzyme in ERK (one of the MAP kinases) signalling and Raf is the primary target of PKC (Kolch, 2000). The Gα<sub>q</sub> pathway is involved in thrombin-induced fibroblast proliferation and differentiation, platelet aggregation and granule secretion (Bogatkevich et al, 2001; Benka et al, 1995; Baffy et al, 1994). Gα<sub>q</sub>-mediated PAR<sub>1</sub> responses on platelets may be particularly relevant *in vivo* since Gα<sub>q</sub>-deficient mice exhibit reduced thrombin-induced aggregation and degranulation (Offermanns et al, 1997). PAR<sub>1</sub>-Gα<sub>q</sub> is responsible for NFκB activation and ICAM-1 transcription in endothelial cells following activation by thrombin (Rahman and MacNee, 1998).

*PAR<sub>1</sub>-Gα<sub>12/13</sub> pathway*

Gα<sub>12/13</sub> has been mainly implicated in the regulation of cytoskeletal structure, cell shape and migration. Following interaction of PAR<sub>1</sub> with Gα<sub>12/13</sub>, guanine-nucleotide exchange factor binding results in the activation of small G-proteins such as Rho GTPases. Rho GTPases belong to the RAS superfamily (which consists of Rho, Rac and Cdc42). These are involved in the control of cell growth and organisation of the actin cytoskeleton (Wennerberg et al, 2005) and mediate the downstream activation of RhoA. PAR<sub>1</sub> is known to couple to Gα<sub>12/13</sub> in platelets, endothelial cells and astrocytoma cells (Aragay et al, 1995; Offermanns et al, 1994; Vouret-Craviari et al, 1998). Thus thrombin mediates platelet shape and cytoskeletal organisation in endothelial cells via the Gα<sub>12/13</sub> pathway (Vouret-Craviari et al, 1998; Klages et al, 1999). Apart from RhoA, PAR<sub>1</sub> coupling to Gα<sub>12/13</sub> activates the Jun-N-terminal kinase pathway, which together with the activation of Gα<sub>q</sub> has been suggested to play role in thrombin-mediated fibroblast transformation (Marinissen et al, 2003). Finally, the PAR<sub>1</sub> Gα<sub>12/13</sub> pathway is responsible for thrombin-stimulated DNA synthesis and cell migration in smooth muscle cells (Seasholtz et al, 1999).

### **1.9.3. Regulation of PAR<sub>1</sub> signalling**

Activation of PAR<sub>1</sub> by proteinases such as thrombin is an irreversible process as cleavage of the N-terminus by the proteinase results in the permanent attachment of the tethered ligand to the receptor thus enabling continuous activation. A number of mechanisms contribute to the cessation of PAR<sub>1</sub> signalling (reviewed in (Ossovskaya and Bunnett, 2004; Dery et al, 1998)).

First, the PAR<sub>1</sub> receptor undergoes rapid intracellular desensitisation leading to the uncoupling of PAR<sub>1</sub> from its downstream signalling cascades and internalisation of the receptor (Ishii et al, 1993). In this process, receptor phosphorylation effectively disrupts the ability of the receptor to interact with G proteins and thus terminates signal transduction. This process is mediated via two classes of protein kinases. Homologous receptor desensitization involves agonist-dependent phosphorylation of specific C-terminal residues of the receptor by G protein receptor kinases, which utilise proteins known as  $\beta$ -arrestins as cofactors. Heterologous receptor desensitization involves agonist-independent activation of a separate receptor C-terminal phosphorylation by other intracellular protein kinases such as PKC and PKA (Yan et al, 1998).

Second, following activation, PAR<sub>1</sub> is recruited into clathrin-coated pits and internalised by endocytosis to be degraded intracellularly (Hoxie et al, 1993). Over 85% PAR<sub>1</sub> is internalised within 1 min in erythroleukemic and megakaryoblastic cell lines (Hoxie et al, 1993) and trafficked to lysosomes for degradation (Brass et al, 1994). This process requires lysosomal sorting which is dependent on the c-terminus of PAR<sub>1</sub> (Brass et al, 1994) and the membrane associated protein, sorting nexin-1 (SN-1) (Wang et al, 2002). A portion of cleaved PAR<sub>1</sub> avoid degradation and traffic back to the cell surface but remain unresponsive to further proteolytic activation (Brass et al, 1994). Receptor endocytosis also occurs independently of receptor activation, and thus PAR<sub>1</sub> is known to cycle constitutively between the plasma membrane and intracellular stores (Shapiro et al, 1996).

The ability of a cell to replenish PAR at the cell surface is dependent on both the mobilization of intracellular receptor stores and the synthesis of new receptors. Abundant intracellular stores of PARs have been reported in endothelial cells (Horvat and Palade, 1995), fibroblasts (Hein et al, 1994) and epithelial cells; although in all cells, receptor replenishment after prolonged exposure to activating proteinases is dependent on the synthesis of new receptors. Platelets lack the ability to synthesize

new receptors and have limited intracellular receptor pools. Thus they are unable to regain responsiveness following their activation by thrombin (Coughlin, 1999).

PAR<sub>1</sub> may also be subject to extracellular inactivation by proteinases such as neutrophil elastase, cathepsin G and proteinase 3. These proteinases have been shown to remove the tethered ligand from PAR<sub>1</sub> by limited proteolysis thus rendering PAR<sub>1</sub> unable to further activation by proteinases (Renesto et al, 1997).

### 1.10. Role of PAR<sub>1</sub> in fibrosis

Since the generation of PAR<sub>1</sub> knock-out mice by Connolly et al in 1996 (Connolly et al, 1997) our understanding of PAR<sub>1</sub> biology has seen rapid advances. The phenotype of the PAR<sub>1</sub><sup>-/-</sup> mouse shows 50% intraembryonic lethality due to a defect in vascular development, however those that survive are grossly normal (Griffin et al, 2001). By far the most significant role of PAR<sub>1</sub> post-development is in mediating platelet aggregation by thrombin. However PAR<sub>1</sub> exerts a variety of different cellular effects that may play an important role in tissue inflammation and repair and may thus contribute to the process of fibrosis (reviewed in (Chambers, 2003)). These will now be discussed in detail.

#### 1.10.1. PAR<sub>1</sub> and inflammation

Activation of PAR<sub>1</sub> by thrombin plays a major role in inducing the release of potent pro-inflammatory cytokines and chemokines from a variety of different cell types including fibroblasts, epithelial cells, monocytes/macrophages and vascular endothelial cells (summarised in **Table 1.6.**, also reviewed in (Bunnett, 2006;Coughlin and Camerer, 2003). These are also released following FXa and TF/FVIIa/FXa complex-mediated activation of PAR<sub>1</sub>. Similarly, a number of different pro-inflammatory mediators may induce tissue factor expression on epithelial cells, macrophages and fibroblasts (Idell et al, 1994), so that it has been proposed that PAR<sub>1</sub> acts as a cellular sensor of tissue injury mediating the cross talk between inflammation and coagulation (Chambers, 2003). Thrombin is a known inducer of endothelial cell adhesion molecule expression, including P-selectin and ICAM-1 *in vitro* (Sugama et al, 1992) and may therefore facilitate the recruitment of inflammatory cells via both chemokine production and adhesion molecule expression.

PAR<sub>1</sub> is highly expressed on endothelial cells and activation of the receptor by thrombin induces endothelial cell permeability and contraction *in vitro* (Bogatcheva et al, 2002). This mechanism may be directly relevant *in vivo* as intravenous infusion of thrombin

increases pulmonary vascular permeability in experimental animal models (reviewed in (Siflinger-Birnboim and Johnson, 2003)). Thus PAR<sub>1</sub> mediated signalling events post lung injury may be a major mechanism by which vascular permeability is increased, allowing the leakage of coagulation proteinases and fibrin into the extravascular space (reviewed in (Idell, 2003)).

Interestingly, recent studies have shown that APC can have barrier protective effects on an endothelial cell monolayer (Feistritzer and Riewald, 2005; Finigan et al, 2005), which at least *in vitro* is thought to be mediated by PAR<sub>1</sub>-dependent transactivation of sphingosine-1 phosphate signalling (Feistritzer and Riewald, 2005). Thus, activation of PAR<sub>1</sub> by either thrombin or APC can mediate both barrier disruption and barrier protection, respectively. However, very low concentrations of thrombin had similar barrier protective effects to APC and very high concentrations of APC were barrier disruptive (Feistritzer and Riewald, 2005), suggesting that the rate of PAR<sub>1</sub> activation determines downstream cellular effects of different PAR<sub>1</sub> activators. Similarly to APC, FXa has now been shown to mediate barrier protection via PAR<sub>1</sub> and also via a PAR<sub>2</sub>-dependent mechanisms (Feistritzer et al, 2005).

### **1.10.2. PAR<sub>1</sub> exerts potent pro-fibrotic effects in vitro**

Research performed over the last ten years in our laboratory and by others has provided compelling evidence that PAR<sub>1</sub> is the main receptor involved in mediating the pro-fibrotic effects of thrombin, including its effects on fibroblast chemotaxis (Dawes et al, 1993), proliferation (Dawes et al, 1993), extracellular matrix production (Chambers et al, 1998) and fibroblast to myofibroblast differentiation (Bogatkevich et al, 2001). More recently, our laboratory has also shown that the stimulatory effects of FXa on fibroblast proliferation and procollagen production are mediated via the activation of PAR<sub>1</sub> rather than PAR<sub>2</sub> (Blanc-Brude et al, 2005). Furthermore, thrombin has also been shown to be a major fibroblast mitogen in BALF fluid from patients with pulmonary fibrosis (Hernandez Rodriguez et al, 1995) (Ohba et al, 1994). In contrast to fibroblast biology, PAR<sub>1</sub> activation on epithelial cells has been implicated in alveolar epithelial cell apoptosis *in vitro* (Suzuki et al, 2005).

The majority of PAR<sub>1</sub>-mediated cellular effects are mediated via the induction and release of a host of secondary mediators. These include several pro-inflammatory and pro-fibrotic mediators, many of which have been implicated in the pathogenesis of pulmonary fibrosis. An extensive list of all mediators released following PAR<sub>1</sub> activation is provided in **Table 1.6**. For example, PAR<sub>1</sub> has been shown to be a potent inducer of

PDGF-AA and also upregulates the expression of the receptor PDGF  $\alpha$ -receptor in fibroblasts. There is good evidence that this is a major mechanism by which PAR<sub>1</sub> mediates its potent mitogenic effects in these cells (Blanc-Brude et al, 2001; Ohba et al, 1994). In addition blocking PDGF is associated with significant protection from experimentally-induced pulmonary fibrosis (Rice et al, 1999; Yoshida et al, 1999). PAR<sub>1</sub> activation leads to the rapid and potent induction of CTGF by cultured lung fibroblasts (Chambers et al, 2000), as well as epithelial cells (Riewald et al, 2001). Interestingly, this growth factor has been shown to mediate both myofibroblast differentiation and collagen deposition via its N-terminal domain and fibroblast proliferation via the C-terminal domain (Grotendorst and Duncan, 2005). There is good evidence that CTGF exerts its effects on these processes depending on the relative presence of the growth factors IGF (promotes CTGF mediated proliferation) and EGF (promotes CTGF-mediated collagen deposition) (Grotendorst and Duncan, 2005).

During the course of the work performed in this thesis, another mechanism by which activation of PAR<sub>1</sub> may promote fibrosis was uncovered by Jenkins et al working in collaboration with our laboratory. The observation was made that PAR<sub>1</sub> ligation can lead to the activation of latent TGF- $\beta$ <sub>1</sub> in alveolar epithelial cells (Jenkins et al, 2006). This mechanism is thought to occur in an  $\alpha$ v $\beta$ 6 integrin-dependent manner and it has been further proposed that this mechanism may contribute to latent TGF- $\beta$ <sub>1</sub> activation following bleomycin-induced lung injury (Jenkins et al, 2006). Interestingly, previous studies in our laboratory revealed that in PAR<sub>1</sub> knock out mice, TGF- $\beta$  immunoreactivity, particularly in fibrotic foci following bleomycin-injury, was decreased. This raised the possibility that PAR<sub>1</sub> may also be important in TGF- $\beta$ <sub>1</sub> production/activation in fibroblasts. The work presented in this thesis has examined this hypothesis in the context of FXa signalling.



Pro-inflammatory mediators	Cellular source
RANTES	Synovial fibroblasts (Hirano et al, 2002)
CCL2	Monocytes/macrophages (Colotta et al, 1994) Endothelial cells (Colotta et al, 1994; Riewald et al, 2002) Dermal fibroblasts (Bachli et al, 2003) Hepatic stellate cells (Fiorucci et al, 2004) HUVEC (Marin et al, 2001)
TNF- $\alpha$	Monocytes/macrophages (Naldini et al, 1998) Microglia (Suo et al, 2002)
IL-1 $\beta$	Monocytes/macrophages (Naldini et al, 2002)
IL-6	Endothelial cells (Johnson et al, 1998) Monocytes (Naldini et al, 2000) Lung epithelial cells (Asokanathan et al, 2002) Lung fibroblasts (Sower et al, 1995) Smooth muscle cells (Kranzhofer et al, 1996)
IL-8	HUVEC (Marin et al, 2001) Lung epithelial cells (Asokanathan et al, 2002) Dermal fibroblasts (Bachli et al, 2003) Lung fibroblasts (Ludwicka-Bradley et al, 2000) Monocytes (Naldini et al, 2000)
PGE <sub>2</sub>	HUVEC (Houliston et al, 2002) Colonic fibroblasts (Seymour et al, 2003) Epithelial cells (Asokanathan et al, 2002)
Pro-fibrotic mediators	Cellular source
PDGF	Alveolar macrophages (Tani et al, 1997) HUVEC (Shankar et al, 1994) Lung fibroblasts (Blanc-Brude et al, 2005) Vascular smooth muscle cells (Stouffer et al, 1993) Lung epithelial cells (Shimizu et al, 2000)
CTGF	Lung fibroblasts (Chambers et al, 2000) Epithelial cells (Riewald et al, 2001)
TGF- $\beta_1$	Vascular smooth muscle cells (Stouffer and Runge, 1998) Platelets (Soslau et al, 1997) Epithelial cells (Jenkins et al, 2006)
bFGF	Vascular smooth muscle cells (Stouffer and Runge, 1998)
Endothelin-1	Endothelial cells (Marsen et al, 1995) Vascular smooth muscle cells (Lepailleur-Enouf et al, 2000) Monocytes (Srivastava and Magazine, 1998)
TSP-1	Endothelial cells (McLaughlin et al, 2005a)
IGF	Vascular smooth muscle cells (Du et al, 2001)

**Table 1.6. Pro-inflammatory and pro-fibrotic mediators induced in response to PAR<sub>1</sub> activation**

### 1.10.3. PAR<sub>1</sub> exerts potent pro-fibrotic effect *in vivo*

In support of a role of PAR<sub>1</sub> in mediating the fibrotic response to lung injury, our laboratory has shown that the immunoreactivity for PAR<sub>1</sub> is highly increased in the bleomycin model of lung injury and fibrosis and is mainly associated with inflammatory and fibrotic foci (Howell et al, 2001). Furthermore PAR<sub>1</sub> immunoreactivity in the human fibrotic lung mirrors that of the bleomycin animal model, as PAR<sub>1</sub> immunoreactivity is very low in normal human lung but highly increased in fibrotic lung samples where it localises to macrophages and fibroblasts within active fibrotic lesions (Howell et al, 2005). More recently, our laboratory has further shown that mice deficient in the PAR<sub>1</sub> receptor are protected from inflammatory cell recruitment, microvascular leak, lung oedema and fibrosis in response to bleomycin injury (Howell et al, 2005). This is associated with a reduction in key pro-inflammatory and pro-fibrotic cytokines, including CCL2/MCP-1 and CTGF which are known to be induced following PAR<sub>1</sub> activation *in vitro* (see **Table 1.6.**). The reduction in the growth factor CTGF is particularly important given the previous observation that protection from bleomycin-induced lung fibrosis by direct thrombin inhibition is also associated with a decrease in CTGF protein expression (Howell et al, 2001). CTGF has been highly implicated in the pathogenesis of pulmonary fibrosis, although the mechanism by which CTGF contributes to the development of pulmonary fibrosis is not well understood. For example, direct adenoviral gene transfer of CTGF into mice lungs only resulted in a transient fibrotic response suggesting that CTGF is not a direct pro-fibrotic mediator in this organ (Bonniaud et al, 2003). However, *in vitro* studies clearly demonstrated pro-fibrotic roles for this growth factor (Grotendorst and Duncan, 2005) and co-administration of CTGF and bleomycin in mice resistant to fibrosis induced a progressive pro-fibrotic response (Bonniaud et al, 2004). In addition, increased amounts of CTGF have been measured in patients with fibrotic lung disease compared with normal patients (Allen et al, 1999). The observation that CCL2/MCP-1 expression is reduced following bleomycin injury in PAR<sub>1</sub> knock-out mice is also of particular interest since CCL2/MCP-1 blockade has been shown to be protective in both bleomycin- and FITC-induced models of pulmonary fibrosis (Gharaee-Kermani et al, 2003; Moore et al, 2001). This may thus represent another potential mechanism by which PAR<sub>1</sub> exerts its pro-fibrotic effects *in vivo*.

### 1.11. Summary and hypothesis

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders, characterised by the excessive deposition of extracellular matrix proteins within the pulmonary interstitium. Current evidence suggests that the differentiation of fibroblasts into highly synthetic and contractile myofibroblasts is central to the pathogenesis of pulmonary fibrosis.

In this chapter, the role of the coagulation cascade and in particular the coagulation proteinase FX in lung pathobiology has been reviewed. Uncontrolled coagulation activity has been documented in the lungs of patients with pulmonary fibrosis, with increased procoagulant activity in the BALF obtained from these patients. FXa has been shown to exert a number of cellular effects and these are mediated via the activation of its major signalling receptors, PAR<sub>1</sub> and PAR<sub>2</sub>.

It is generally held that coagulation zymogens are principally synthesized in the liver and released into the circulation as inactive precursors which are only activated as a consequence of the initiation of the tissue factor (TF)-dependent coagulation pathway at sites of injury. However, recent global expression profiling studies performed in our laboratory revealed that the FXa precursor, zymogen Factor X (FX), is locally upregulated in response to bleomycin-induced fibrosis in the murine lung (Scotton et al, unpublished observation).

This thesis will therefore address the following hypothesis:

***FX/FXa is locally expressed in the fibrotic lung and contributes to the development of pulmonary fibrosis by influencing fibroblast function.***

The specific aims of this thesis are to:

- (i) Confirm the local upregulation of FX in the fibrotic lung by assessing FX mRNA and protein levels in the bleomycin model of lung injury and fibrosis and in human IPF samples.
- (ii) Determine if FXa influences fibroblast to myofibroblast differentiation and identify the signalling receptor involved and the potential role of TGF- $\beta_1$  in mediating this effect.
- (iii) Evaluate the role of FXa in the development of pulmonary fibrosis *in vivo*, by assessing the effect of the potent and selective direct FXa inhibitor, ZK 807834 on lung collagen accumulation in the bleomycin model of lung injury and fibrosis.

## Chapter 2: Materials & Methods

### Materials

#### 2.1. Chemicals, solvents and tissue culture materials

All chemicals were of analytical grade or above and obtained from *Sigma Aldrich (UK)* unless stated otherwise. All water used for the preparation of buffers was distilled and deionised using a Millipore Water Purification system (*Millipore R010 followed by Milli-Q Plus; Millipore Ltd., UK*). Solvents used for the preparation of high pressure liquid chromatography (HPLC) buffers and solutions were of HPLC grade and obtained from *BDH-Merck Ltd. (UK)*. Sterile tissue culture flasks and plates, polypropylene centrifuge tubes and pipettes were all obtained from *Nunc (Denmark)*. All sterile tissue culture media, sterile tissue culture grade trypsin/EDTA and antibiotics were obtained from *Invitrogen (UK)*. Foetal calf serum (FCS) was purchased from *Invitrogen (UK)*.

#### 2.2. Coagulation factors, cytokines, growth factors and peptides

Purified human FXa was purchased from *Enzyme Research Labs Ltd. (UK)*; human  $\alpha$ -thrombin was obtained from *Sigma-Aldrich (UK)*; human TGF- $\beta_1$  and TNF- $\alpha$  were purchased from *R&D Systems (UK)*. Bleomycin was purchased from *Kyowa-Hakka (USA)*. The specific PAR<sub>1</sub> agonist peptide TFLLR-NH<sub>2</sub> and the corresponding control peptide FTLLR-NH<sub>2</sub> were synthesized and provided by Professor R. Mecham (University of Washington, MO, USA). The PAR<sub>2</sub> agonist peptide, SLIGKV-NH<sub>2</sub> was purchased from *Auspep (Australia)*. All preparations were dissolved in DMEM culture medium, aliquotted and stored at -80 °C.

#### 2.3. Inhibitors and Antibodies

The specific thrombin inhibitor, recombinant leech hirudin (rHir), was purchased from *Sigma-Aldrich (UK)*. The selective PAR<sub>1</sub> antagonist RWJ-58259 was a kind gift from Dr Claudia Derian (*Johnson and Johnson Pharmaceutical Research and Development, USA*). The specific FXa inhibitor, antistasin core peptide D-Arg32-Pro38, was purchased from *Bachem (UK)*. Pertussis toxin was obtained from *Merck Chemicals (UK)*. The Q94 compound was a kind gift from Annette Gilchrist, (*Caden Biosciences, WI, USA*). The TGF- $\beta_1$  type I receptor kinase (ALK5) inhibitor, SB431542, was purchased from *Sigma-Aldrich (UK)*. The ALK5 inhibitor SD-208 was a kind gift from Dr. Linda Higgins (*Scios, CA, USA*). The thrombospondin-1 (TSP-1) inhibitory peptide LSKL and the scrambled control peptide SLLK were a kind gift from Professor Murphy-Ullrich (*University of Alabama, AL, USA*).

The small molecule inhibitor of FXa activity, ZK 807834, was a kind gift from Dr John Morser (*Berlex Inc., CA, USA*).

The antibodies used for Western blotting were as follows. A mouse polyclonal anti- $\alpha$ -SMA (*clone 1A4*) was purchased from *Sigma-Aldrich (UK)* (Skalli et al, 1986). Mouse monoclonal anti-TGF- $\beta$  (*clone 1D11*) was from *R&D Systems (UK)* (Dasch et al, 1989). The rabbit polyclonal anti-FX antibody was obtained from *Santa Cruz Biotechnology (Germany)* (Lenzerini et al, 1981)- this antibody recognizes the heavy chain of FX and showed crossreactivity with FXa on western blotting. The mouse polyclonal anti-TSP-1 antibody was purchased from *Neomarkers (USA)* (Dixit et al, 1986) - this antibody only recognises the reduced form of TSP-1. The rabbit polyclonal anti-phosphoSmad2/3 (Heldin et al, 1997) and anti-Smad2 (Heldin et al, 1997) total were purchased from *Cell Signaling Technology (USA)*. The goat polyclonal anti-ERK2 antibody was purchased from *Santa Cruz Biotechnology (Germany)* (Boulton et al, 1991). ERK2 was used as a loading control to ensure equal protein loading on SDS-PAGE; it was chosen above traditional loading controls such as  $\beta$ -actin or total actin since the level of these proteins changed upon FXa stimulation of fibroblasts.

Antibodies used for immunohistochemical localization studies were as follows. Rabbit polyclonal anti-FX (Lenzerini et al, 1981) was purchased from *Santa Cruz Biotechnology (Germany)*. The goat polyclonal anti-TSP-1 (Riewald and Ruf, 2005) was purchased from *Santa Cruz Biotechnology (Germany)*. The rabbit polyclonal anti- $\alpha$ -SMA was purchased from *Lab Vision (Fremont, California)*. The rabbit polyclonal anti-TRED PAR<sub>1</sub> antibody was a generous gift from Professor Eleanor Mackie (University of Melbourne Australia).

#### **2.4. Alveolar and bronchial epithelial cells, fibroblasts and tMLEC**

A human type II alveolar epithelial cell line (A549), human bronchial epithelial cell line (BEAS-2B) and human foetal lung fibroblast cell line (HFL-1) were obtained from the *American Type Culture Collection (ATCC)* and were used at no more than passage 20. Although the A549 cell line was originally obtained through explant culture of lung carcinoma tissue it is nevertheless widely used as a model of alveolar epithelial cell behaviour as it expresses all the characteristic markers of this cell type (Lieber et al, 1976).

Primary cultures of human adult lung fibroblasts (pHALFs) were a kind gift from Dr. Robin McNulty in our laboratory, and previously showed high responsiveness to stimulation by coagulation proteinases (Chambers et al, 2000). Primary fibroblasts were grown from 1mm<sup>3</sup> explants dissected from normal human lung tissue. The explants were cultured in DMEM supplemented with 10% FCS. The culture medium was replaced with fresh medium one day after isolation and every three days thereafter for three weeks. Eventually, fibroblasts were collected by trypsinisation and then characterised morphologically and by differential immunocytochemical staining for a selection of smooth muscle cell, endothelial and fibroblast markers such as  $\alpha$ -SMA, von Willebrand factor, vimentin and myosin. Primary cells were discarded after 5 to 10 passages.

Wild-type mouse lung fibroblasts and PAR<sub>1</sub>-deficient mouse lung fibroblasts were a kind gift from Dr P. Andrade-Gordon (*R.W. Johnson Pharmaceutical Research Institute Spring House, PA, USA*). These fibroblasts were immortalised through SV40 transformation and used between passage 20 and 35 without any noticeable alteration in phenotype.

Transformed mink lung epithelial reporter cells (tMLEC) stably expressing firefly luciferase under the control of a TGF- $\beta$ -sensitive portion of the plasminogen activator inhibitor-1 promoter were a gift from Dr Dan Rifkin (*Department of Cell Biology, New York University School of Medicine, NY, USA*) and have been previously described (Abe et al, 1994).

Fibroblasts deficient in G $\alpha_{q11}$  were isolated from G $\alpha_{q11}$ -deficient mouse embryos at embryonic day 10.5 and were a kind gift from Professor Stefan Offermanns (*Pharmakologisches Institut, University of Heidelberg, Germany*) and have been previously described (Zywietz et al, 2001).

## Methods

### 2.5. Animals

Male C57Bl/6J mice (*Harlan UK Ltd., UK*) were kept at the Central Biological Services Unit, University College London. Animals were housed in a specific pathogen-free facility in individually-ventilated cages, with free access to food and water (12h light/dark cycle, normal sodium dry fishmeal diet, temperature 18-20 °C). All procedures were performed on mice between 8 and 10 weeks of age, in accordance

with the UK Home Office Animals Scientific Procedures Act. Animals were weighed prior to investigation and then daily for the duration of the experiment.

## **2.6. Animal Model of Pulmonary Fibrosis**

### **2.6.1. Oropharyngeal instillation of bleomycin**

Bleomycin (1 or 2mg/kg body weight in 50µl of saline) or saline was administered by oropharyngeal installation as described previously by Lakatos *et al* (Lakatos *et al*, 2006). Following light halothane-induced anaesthesia, mice were hung from an elastic band by their teeth, the nose was pinched shut, and the tongue was held (to prevent the swallow reflex) - forcing the mouse to breathe through its mouth. Consequently, saline/bleomycin introduced to the back of the animal's mouth was aspirated. Mice were later sacrificed by intraperitoneal injection of pentobarbitone, and severing of the abdominal inferior vena cava.

For measurement of total lung collagen, real time RT-PCR and immunoblotting, lungs were removed, blotted dry and the trachea and major airways were excised before the separated lobes were immediately snap frozen in liquid nitrogen, weighed and pulverized under liquid nitrogen. For histological and immunohistochemical analysis, the trachea was cannulated and lungs were insufflated with 4% paraformaldehyde in PBS at a pressure of 25cm H<sub>2</sub>O, followed by removal of the heart and inflated lungs *en bloc* and immersion for 4 hours in fresh fixative. Subsequently, lungs were transferred to 15% sucrose in PBS and left overnight at 4°C before transfer to 70% ethanol. For BALF analysis, the trachea was cannulated and normal saline was instilled in 0.5ml aliquots over 15 seconds, left *in situ* for 30 seconds, and withdrawn over 15 seconds and stored in polypropylene tubes on ice. The procedure was repeated five times and greater than 90% of the total instillate was recovered. Lung tissue homogenates were prepared based on a method described by (Keane *et al*, 1999). Frozen lung powder was mixed with 0.5ml PBS with added protease inhibitors (Complete Mini, *Roche Diagnostics, UK*) in polypropylene tubes. Samples were homogenised (3 x 20 seconds on ice) with a Polytron PT1035 mechanical homogeniser (*Kinematica GmbH, Switzerland*), filtered through a 1.2µm filter (*Sartorius, Germany*) and aliquots were frozen at -80°C until use.

### **2.6.2. Aerosolized instillation of bleomycin**

This bleomycin model was performed by Dr Königshoff in Professor Eickelberg's laboratory in Giessen, Germany. The obtained lung material was then subsequently

provided for analysis. Six to eight week-old pathogen-free female C57BL/6N mice (*Charles River, Germany*) were used throughout this study. All experiments were performed in accordance with the guidelines of the Ethic's Committee of University of Giessen School of Medicine and approved by the local authorities. Mice had access to water and rodent laboratory chow *ad libitum*. Bleomycin sulphate (*Almirall Prodesfarma, S.A.*) was dissolved in sterile saline solution and applied by microspray as a single dose of 0.08mg/mouse in a total volume of 200 $\mu$ l. Control mice received 200 $\mu$ l saline. The lungs from mice sacrificed after 7, 14, or 21 days of bleomycin exposure were harvested and immediately snap-frozen in liquid nitrogen.

## 2.7. BALF FXa activity

BALF samples were obtained using the method described in **Section 2.6.1.** and centrifuged at 300g for 10 minutes at 4°C. FXa activity was measured using a commercially-available spectrophotometric assay for FXa (*American Diagnostica, USA*). A standard curve was generated by diluting stock FXa (1U/ml; certified value 800 $\mu$ g/100U) in PBS. A 25 $\mu$ l aliquot of each dilution or undiluted BALF sample was pipetted into a 96-well plate and 25 $\mu$ l of chromophore S222 (1mM, Methoxycarbonyl-D-cyclohexylglycyl-arginine-para-nitroanilide acetate, Spectrozyme® FXa, American Diagnostica Inc., USA) was added to each sample and incubated at 37°C. FXa converts this chromophore into the chromogen, p-nitroaniline (pNA). The enzymatic activity of FXa was thus detected by measuring the increase in absorbance of the free chromophore p-nitroaniline (pNA) generated per unit time at  $\lambda_{405\text{ nm}}$ .

Serial measurements were read on a kinetic recording spectrophotometer, every minute for three hours. Absorbance for each FXa dilution was plotted graphically against time. The rate of change in absorbance was then calculated over the initial linear part of the curve. FXa concentrations in each sample were then calculated by extrapolating values from the standard curve.

## 2.8. FXa inhibition *in vivo* using ZK 807834

The *in vivo* contribution of FXa to lung collagen accumulation was investigated using the direct FXa inhibitor ZK 807834 in the bleomycin model of fibrosis induced by oropharyngeal instillation of bleomycin (described in **Section 2.6.1.**). Twenty-four hours post-bleomycin, administration of ZK 807834 (50mg/kg in acidified saline) or vehicle was started, by intraperitoneal injection twice a day in the first week and once a day in



the second week until the end of the experiment (14 days). This regime was chosen because this dosing gave a circulating concentration of ZK 807834 above  $2\mu\text{M}$ , which is sufficient to double the prothrombin time in C57Bl/6J mice (data provided by Berlex). Mice were subsequently sacrificed and total lung collagen was determined by measuring hydroxyproline content in aliquots of pulverized lung (described in **Section 2.9.**). During the course of the ZK 807834 experiment, three mice in the saline/ZK 807834 group (originally  $n=6$ ) and three mice in the bleomycin/ZK 807834 group (originally  $n=9$ ) were sacrificed due to intraperitoneal haemorrhage arising from the multiple intraperitoneal injections. In addition, one mouse in the bleomycin/vehicle group (originally  $n=8$ ) was excluded from the HPLC analysis as an outlier, because it did not display the characteristic loss of body weight following bleomycin instillation or any increase in lung weight.

## **2.9. Determination of total lung collagen**

Total lung collagen was determined by measuring hydroxyproline (Hyp) content in aliquots of pulverised lung as described previously (Campa et al, 1990; Chambers et al, 1994), and assuming that lung collagen contains 12.2% w/w hydroxyproline (Laurent et al, 1981). Hydroxyproline was quantified by reverse-phase high performance liquid chromatography (HPLC) of 7-chloro-4-nitrobenzo-oxa-1,3-diazole (NBD-Cl)-derivatised acid hydrolysates. Secondary amino acids such as hydroxyproline react with NBD-Cl to generate a chromophore with maximum light absorbance at 495nm. NBD-Cl also reacts with primary amino acids but these amino acids only have limited absorbance at these wavelengths. In addition the reactions with Hyp and proline occurs one order of magnitude faster than with primary amino acids (Ahnoff et al, 1981). Interference from primary amino acids is therefore minimised by keeping the derivatisation time to 20 min, a timepoint at which the extent of Hyp derivatisation with NBD-Cl at  $37^\circ\text{C}$  was previously shown to be maximal for up to 20nmol Hyp (Campa et al, 1990).

### **2.9.1. Pre-Column Derivatisation**

For each sample, approximately 20mg of lung powder was accurately weighed, and hydrolysed in 2ml 6M HCl for 16hours at  $110^\circ\text{C}$  in a pyrex tube. Hydrolysates were decolourised with activated charcoal, filtered through a  $0.65\mu\text{m}$  filter (*Millipore Ltd., UK*), and diluted 1 in 100. 200 $\mu\text{l}$  aliquots of each hydrolysate were transferred to a microfuge tube and evaporated to dryness under vacuum on a Speedvac (*Thermo Electron Corporation, UK*). The resulting residue was re-dissolved in 100 $\mu\text{l}$  HPLC-grade water, buffered with 100 $\mu\text{l}$  of 0.4M potassium tetraborate (pH 9.5) and reacted

with 100µl 36mM NBD-Cl (in methanol) to a final concentration of 12mM NBD-Cl. The samples were then incubated in a hot block at 37°C for 20 minutes. The reaction was stopped by the addition of 50µl 1.5M HCl. At this point 150µl of 3.33x Buffer A was also added (Buffer A is described in **Table 2.1.**). Samples were then filtered through an HPLC low dead-volume filter (pore size 0.22µm, type GV; *Millipore Ltd, UK*) into a polypropylene insert within an Amber Snap Seal vial (*Laboratory Sales Ltd., UK*). These vials were then loaded onto the HPLC apparatus and the samples were sequentially injected onto the HPLC column and eluted with an acetonitrile gradient as described below.

### **2.9.2. Instrumentation and chromatography conditions**

The HPLC apparatus employed for these measurements was a Beckman System Gold HPLC (*Beckman Coulter, UK*) with a reverse-phase cartridge column (LiChroCART LiCrospher, 250mm length x 4mm diameter, 5µm particle size, 100 RP-18; *BDH/Merck, UK*) protected by a directly coupled pre-column (LiChrosorb, 4mm x 4mm, 5µm, 100 RP-18; *BDH/Merck*). Columns were continuously maintained at 40°C in a heated column oven. At the beginning of each experiment, the running buffers were degassed with helium (*BOC Ltd, UK*) and the HPLC system equilibrated in running buffer A for 40 minutes. The first two samples derivatised were Hyp standard solutions (equivalent to 50 pmol/L Hyp, *Sigma*) which were then used for calibration. NBD-Cl derivatives in samples and standards were eluted with an acetonitrile gradient, which was achieved by changing the relative proportions of running buffers A and B over time. Chromatographic conditions and buffers employed in this process are summarised in **Table 2.1.**

Post-column detection was achieved by monitoring absorbance at 495nm using a flow-through variable wavelength monitor. Hyp elutes as a discrete peak between five and seven minutes following its injection onto the column, between glutamine (3.5 minutes) and serine (seven to nine minutes) and just prior to the mobile phase becoming predominantly organic. Remaining amino acid derivatives in the sample were eluted as the hydrophobicity of the acetonitrile organic buffer was increased. The column running and regeneration time was 25 minutes.

Quantification of the hydroxyproline content in each 100 µl sample injected into the column was determined by comparing peak areas of chromatograms obtained for each sample to those generated from the standard solutions derivatised and separated

under identical conditions at the beginning of each experiment. Total lung collagen was expressed as mg/lung and derived from the equation:

$$\text{Total lung collagen} = \text{HPLC peak area} \times 10 \times 100 \times 5 \times 1/1000 \times 100/12.2 \times 131/1000000 \times \text{lung weight/powder weight}$$

<b>Column</b>	<b>LiChrospher, 100 RP-18, 250 x 4mm, 5µm</b>	
<b>Mobile phase</b>	<b>Buffer A</b> - aqueous acetonitrile (8% v/v) 50mM sodium acetate, pH 6.4	
	<b>Buffer B</b> - aqueous acetonitrile (75% v/v)	
<b>Column flow rate</b>	1.0 ml/min	
<b>Column temperature</b>	40°C	
<b>Detection wavelength</b>	495nm	
<b>Elution gradient</b>	Time (min)	% Buffer B
	0	0
	5	5
	6	80
	12	80
	12.5	0
	25	0

**Table 2.1. Conditions and buffers for the separation of hydroxyproline by reverse-phase HPLC**

## 2.10. RT-PCR and real-time RT-PCR analysis

### 2.10.1. Precautions taken to prevent RNA degradation

For all experiments involving RNA isolation, deionised water was pre-treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) overnight at room temperature; the treated water was then autoclaved to inactivate the DEPC. All subsequent reagents were made from molecular biology grade chemicals DEPC-treated deionised water. All equipment was cleaned thoroughly using RNaseZap (*Sigma Aldrich, UK*), and nuclease-free pipette tips (*Continental Lab products, UK*) were used throughout the extraction process.

### 2.10.2. RNA extraction

Total RNA from frozen powdered lung tissue or cell cultures was isolated with TRIzol reagent as per the manufacturer's protocol (*Invitrogen, UK*). TRIzol is a solution of phenol and guanidine isothiocyanate which disrupts cell membranes by dissolving cell components but maintaining the integrity of RNA. Briefly, 1ml of TRIzol was added to 50-100 mg of lung tissue and this was further homogenised through a 25 gauge needle attached to a 1ml syringe. For isolation from cell cultures, 1ml of TRIzol was added to each well of cells that were grown in a 6-well plate and the well was scraped with a 1ml pipette tip and solution transferred to a 1.5ml eppendorf tube. Subsequently samples were left at room temperature for 5 minutes after which 200µl of chloroform was added to disrupt the cell suspension. Each tube was vortexed and left at room temperature for 10 minutes to allow the solution to separate into an upper aqueous and a lower organic phase. Tubes were then centrifuged at 13,000 rpm for 15 minutes at 4°C in a microfuge and the upper aqueous phase containing the RNA was transferred to a fresh tube with 0.5 ml isopropanol. Tubes were left at room temperature for 10 minutes to allow the RNA to precipitate and were then centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatants in each tube were discarded and 900 µl of 75 % ethanol (*BDH VWR International, UK*) were added to the pelleted RNA and centrifuged at 13,000 rpm for a further 15 minutes at 4°C. The supernatants in each tube were discarded and pellets were air dried and resuspended in an appropriate volume of nuclease-free water (*Ambion, UK*).

### 2.10.3. DNase treatment

Total RNA was DNase-treated to remove contaminating genomic DNA, using an *Ambion* DNase-free kit. This kit comprised a 20 µl reaction in which total RNA, DNase I and RNase inhibitor were added and left to incubate at 37°C for 20 minutes. The reaction was then stopped by adding "inactivating reagent"- a resin which binds the DNase. This was then removed by centrifugation.

RNA quality was subsequently analysed by running samples on an agarose gel: a mixture of 1 µl of total RNA and 11 µL DEPC-treated water was mixed with 3 µl of loading buffer (48 % deionised formamide [*Gibco BRL, UK*], 6 % formaldehyde [*BDH, UK*], 5 % glycerol, 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA pH 8.0 made up in DEPC-treated water). Each RNA sample was heated to 65 °C for 5 minutes and placed on ice prior to loading. The RNA was analysed by electrophoresis on a 1 % agarose-formaldehyde gel (6 % formaldehyde, 1 % agarose, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA pH 8.0).

Images of the RNA were captured with a Syngene Gene Genius Bio-imaging system (*Synoptics, UK*). A ratio of approximately 2:1 of the intensities of the 28S rRNA to the 18S rRNA bands confirmed that the RNA was not significantly degraded. The RNA concentration and protein contamination in each sample was assessed on an Ultrospec 3000 spectrophotometer (*Amersham Biosciences, UK*) by measuring the  $A_{260}$ , and the  $A_{260}/A_{280}$  ratio, respectively.

#### **2.10.4. cDNA synthesis**

cDNA was prepared by reverse transcription (RT) using a RT-PCR kit from *Applied Biosystems, UK*. Reactions were performed according to the manufacturer's instructions. Briefly, a mix was prepared containing a maximum of 1 µg of total RNA, and incubated at room temperature for 10 minutes, 42 °C for 15 minutes, 99 °C for 5 minutes and then 5 °C for 5 minutes:

- a) 5 mM MgCl
- b) 1x PCR Buffer
- c) 1 mM dNTP mix
- d) 2.5 µM random hexamers
- e) 1 µg RNA
- f) 1 U/µl RNase Inhibitor
- g) 2.5 U/1 µl reverse transcriptase

#### **2.10.5. Primer design**

The primers were designed with the help of Dr Chris Scotton and using internet based software. The Ensembl database (<http://www.ensembl.org/>) was used to locate accession numbers for the genomic DNA and RNA and then Spidey (<http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/>) was used to align the genomic and RNA sequences in order to locate intron/exon boundaries. When possible, primers were designed to span introns, eliminating the possibility of amplifying genomic DNA. The mRNA sequence was copied into the primer-designing software, Primer3 ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). The program's parameters were set at: product size 85-130 bp; primer size 18-22 nucleotides long; primer melting temperature 58 °C to 62 °C with an optimum of 60 °C and a maximum temperature difference of 0.5 °C; primer GC % was 40 % to 60 % with an optimum of 50 %; maximum self-complementarity was set at 6.0 and maximum 3' self-complementarity of 2.0 and finally, the maximum poly-X was set at 3 to avoid runs

of nucleotides. The primers were then selected from the list and run *in silico* using FastPCR software. A BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) was also performed to check that the forward and reverse primers were specific for the intended sequence. The primers were manufactured by *Invitrogen, UK*. Primers can be seen in **Table 2.2**.

Gene	Forward primer	Reverse primer
h TGF- $\beta_1$	5'-AAGGGCTACCATGCCAACT-3'	5'-CCGGGTTATGCTGGTTGTA-3'
m FX	5'-CAGCGGTTACTTCCTGGGTA-3'	5'-GCCACAGACCTCTTCCTACG-3'
h FX	5'-GCCCACTGTCTCTACCAAGC-3'	5'-CTTGATGACCACCTCCACCT-3'
h PAR <sub>1</sub>	5'-CCATCGTTGTGTTTCATCCTG-3'	5'-GACCCAAACTGCCAATCACT-3'
h PAR <sub>2</sub>	5'-CACCATCCAAGGAACCAATAG-3'	5'-TGCAGAAAACATCCACAGA-3'
h $\alpha$ -SMA	5'-ATCCTGACTGAGCGTGGCTATT-3'	5'-GGCCATCTCATTTTCAAAGTCC-3'
m/h18s	5'-TTGACGGAAGGGCACCACCAG-3'	5'-GCACCACCACCCACGGAATCG-3'
h TSP-1	5'-CAATGAACGGGACAACCTGC-3'	5'-ATCTCCAATGCGGTCTGAGT-3'
h HPRT	5'-GGCTTTGTATTTTGCTTTTCCA-3'	5'-AAGGACCCACGAAGTGTTG-3'

**Table 2.2. Primers used for real-time RT- PCR**

h=human, m=murine

### 2.10.6. RT-PCR

RT-PCR was performed using a tetrad (*PTC-225, Peltier Thermal cycler, Global Medical Instrumentation, USA*). For each primer pair, a master mix was prepared containing all reagents except the cDNA. The final volume of each PCR reaction was 25  $\mu$ l, containing cDNA, AmpliTaq DNA polymerase, buffer, dNTPs, magnesium chloride (all from *Applied Biosystems, UK*) and 0.25  $\mu$ M of each primer. The following cycling conditions were used: 94 °C 5 minutes/ 35 cycles of 94 °C for 30 seconds/ 60 °C for 30 seconds/ 72 °C for 30 seconds/ 72 °C for 7 minutes and 25 °C for 30 seconds.

20  $\mu$ l of each PCR reaction was added to 5  $\mu$ l of loading buffer (40 % w/v sucrose; 0.25 % w/v bromophenol blue; 0.25 % w/v xylene cyanol, made up in distilled water) and electrophoresed through a 2 % agarose gel containing 0.5  $\mu$ g/ml ethidium bromide and

TBE (90 mM tris, 90 mM borate and 2 mM EDTA). Bands were visualised by UV transillumination. Their sizes were estimated using a co-migrated DNA size marker from *Roche Diagnostics*.

#### **2.10.7. Real-time RT-PCR**

Real time RT-PCR was conducted using the Platinum SYBR Green qPCR SuperMix UDG (*Invitrogen, UK*) with 1ng of cDNA and forward and reverse primers each at a final concentration of 500nM, on a LightCycler 1.5 Real-time Detection System (*Roche, UK*) and analysed using LightCycler Real-time PCR Detection System Software Version 3.5. Cycling conditions were as follows: one cycle of 50 °C (2 minutes), 95 °C (2 minutes); 45 cycles of 95 °C (5 seconds), 55 °C (5 seconds), 72 °C (15 seconds). For laser capture microdissected samples quantitative PCR was performed using an Applied Biosystems Sequence Detection System 7700 (*PE Applied Biosystems, CA, USA*).

The magnesium concentration for each primer set was then optimised. A number of real time RT-PCR reactions were performed in parallel, using template cDNA from a sample known to express the gene of interest but substituted with varying concentrations of  $Mg^{2+}$ . Reactions with the steepest logarithmic amplification plot, and typically also the lowest cycle number at which detectable amplification occurred, indicated the optimal  $Mg^{2+}$  concentration for use with the given primer set.

The efficiency of each primer pair was also assessed by determining crossing point (Cp) values for RT-PCR reactions using a series of half logarithmic dilutions of template cDNA. Cp values were defined as the earliest point of the linear region of the logarithmic amplification plot reaching a threshold level of detection. Log concentrations of samples were plotted against Cp values, and the slope of the plot determined. Efficiency was then given by the equation:  $\text{Efficiency} = 10^{(-1/\text{slope})}$ . Primers were only used if the PCR efficiency was greater than 95%.

To examine the quantitative differences in the mRNA target gene expression in each sample, Cp values were determined from the linear region of the logarithmic amplification plot. Each sample was also tested for expression of an appropriate "housekeeping" gene, either 18S, HPRT or PDGB - the Cp value of which was used to normalise between samples. Fold change was subsequently calculated using the standard  $2^{-\Delta\Delta C_p}$  approach. The specificity of the products obtained by PCR was

confirmed by melting curve analysis, demonstrating a single melting curve indicative of a single PCR product. Statistical analysis was performed using the  $\Delta C_p$  values.

## **2.11. Histological Analysis**

### **2.11.1. Preparation of slides**

Individual lobes of mouse lungs or human biopsy material were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. Before immunostaining, 5  $\mu$ m-thick lung tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS.

### **2.11.2. Immunohistochemical localisation of FX, TSP-1, PAR<sub>1</sub> and $\alpha$ -SMA**

Antigens were unmasked by microwaving sections in 10mM citrate buffer, pH 6.0 (2 x 10 mins) before washing in PBS and incubation with normal goat serum (DAKO, UK) for FX, PAR<sub>1</sub> and  $\alpha$ -SMA or with normal rabbit serum for TSP-1. Antigens were localized by overnight incubation with rabbit anti-FX (1:100 dilution), goat anti-TSP-1 (1:250), rabbit anti-PAR<sub>1</sub> (1:1000) or rabbit anti- $\alpha$ -SMA (1:400) primary antibody. Sections were then washed in PBS, and incubated with a biotinylated goat anti-rabbit or rabbit anti-goat secondary antibody (1:200 dilution) for 1 hour, before washing again in PBS. Sections were incubated with a streptavidin/peroxidase complex (1:200 dilution) for a further 30 minutes, followed by a solution of 600 $\mu$ g/ml of 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories, UK) for colour development. Sections were washed and counterstained using Gill-2 haematoxylin (Thermo-Shandon, USA), dehydrated through a series of incubations in increasing concentrations of ethanol and then xylene, and mounted using a Sakura Coveraid automatic coverslipping machine and Tissue-Tek coverslipping film (Bayer Diagnostics, UK). Specificity of the signal obtained was confirmed by incubating control sections with an isotype-specific, nonimmune rabbit or goat IgG primary antibody (DAKO, UK). Sections were then visualized by microscopy (Leica DM5000B microscope, Leica Microsystems, Germany) and images were captured using a Qicam 12-bit colour fast camera using Q capture software version 2.81 (both from QImaging Corporation, Canada).

### **2.11.3. Demonstration of lung architecture**

Two different staining methods were used to visualise the lung architecture. A modified trichrome staining method (Martius Scarlet Blue (MSB)) was used to highlight collagen deposition as a readout of lung fibrosis. This staining method is based on the use of



differential permeability of tissues to dyes of differing molecular size. The large Chicago Sky Blue 6GX dye permeates collagen fibrils causing areas of fibrosis to appear blue. In order to enable appreciation of inflammatory cell infiltration, a heamatoxylin and eosin method was used. Automated staining of sections was performed using a Sakura DRS 601 Diversified Stainer (*Bayer Diagnostics*).

## **2.12. Patient samples**

### **2.12.1. Giessen cohort**

Lung tissue biopsies were obtained from ten patients with IPF (usual interstitial pneumonia (UIP) pattern; mean age  $51.3 \pm 11.4$  years; 4 females, 6 males) and ten control subjects (organ donors, mean age  $47.5 \pm 13.9$  years; 5 females, 5 males). The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol.

### **2.12.2. Brompton cohort**

Lung biopsy specimens were obtained from 7 patients with IPF (6 male and 1 female, mean age  $55.3 \pm 10.6$ ; 5 specimens obtained at diagnostic surgical lung biopsy and 1 from lung explanted at transplant) and 6 control patients (4 male and 2 female, mean age  $65.3 \pm 15.8$ ; obtained from uninvolved tissue during cancer resection surgery). All biopsies in this study were classified using the diagnostic criteria of the American Thoracic Society/European Respiratory Society consensus (American Thoracic Society/ European Respiratory Society International multidisciplinary consensus classification of the idiopathic interstitial pneumonias, 2002) a pattern of “usual interstitial pneumonia (UIP)”. Approval for the use of material was obtained from the Royal Brompton, Harefield, NHLI and the UCL/UCLH ethics committee. Informed consent was obtained from each subject.

### **2.12.3. Pittsburgh cohort**

Pittsburgh Tissue Microarrays (TMA) were provided by Dr Naftali Kaminski, University of Pittsburgh and were generated as previously described (Kajdacsy-Balla et al, 2007) from formalin-fixed paraffin embedded tissues, stored at the University of Pittsburgh Tissue Bank at the Tissue Array Core facility. The TMA included 12 samples with sarcoidosis, seven samples with hypersensitivity pneumonitis, 18 samples with UIP, as well as three samples from head and neck tumours. Use of stored de-identified tissues has been approved by the University of Pittsburgh Institutional Review Board.

### **2.13. Laser-capture microdissection**

Microdissection was performed by Dr Melanie Königshoff in Professor Oliver Eickelberg's laboratory, Giessen, Germany and was described previously (Fink et al, 1998; Fink et al, 2000b). In brief, 10µm cryosections were mounted on glass slides, stained with hemalaun for 45 seconds, immersed in 70% and 96% ethanol, and stored in 100% ethanol until use. Alveolar septae were selected and microdissected with a sterile 30G needle under optical control using the Laser Microbeam System (P.A.L.M., Bernried, Germany). Microdissected tissues were then transferred into reaction tubes containing 200µl TRIzol and samples processed for RNA analysis, as described in **Section 2.10.**

### **2.14. Alveolar epithelial cell isolation**

Primary alveolar type II epithelial (ATII) cells were isolated from saline- and bleomycin-treated mice by Dr Melanie Königshoff. Lungs were lavaged with 2 x 1 ml sterile PBS, then the tissue was minced with scissors and digested with dispase (2 mg/ml for 60 mins). The suspension were sequentially filtered through 100-, 20-, and 10-µm nylon meshes and centrifuged at 200g for 10 min. The pellet was resuspended in DMEM and a negative selection for lymphocytes/macrophages was performed by incubation on CD16/32- and CD45-coated petri dishes for 30 min at 37°C. Negative selection for fibroblasts was performed by adherence (45 min) on cell culture dishes. Cell purity was routinely assessed by epithelial cell morphology and immunofluorescence analysis of panCK, pro-SPC (both positive),  $\alpha$ -SMA, and CD45 (both negative). Freshly isolated ATII cells were used for further analysis.

### **2.15. Fibroblast isolation**

Lungs from saline- or bleomycin-treated mice, or from tissue samples from IPF/donor lungs, were minced into small pieces and incubated in a solution of human dispase (2 mg/ml) for 60 min. Pieces were plated out and outgrowing fibroblasts were purified by passaging and cultured for about 2 weeks. Identification of fibroblasts was based on the presence of vimentin, collagen and  $\alpha$ -SMA staining. Cells were used between passages 2-4. Cells were maintained in DMEM supplemented with 10% FCS, in a humidified atmosphere containing 5% CO<sub>2</sub>.

## 2.16. Cell culture conditions

HFL-1, pHALFs, mouse lung fibroblasts (MLF), A549, BEAS-2Bs and HepG2s were maintained using standard cell culture techniques. HFL-1, pHALFs, MLF and HepG2s were grown in Dulbecco's modified eagle's medium (DMEM), supplemented with penicillin (200 units/ml), streptomycin (200 units/ml), glutamine (4 mM) and 10% (v/v) FCS. BEAS-2Bs were grown in Optimem supplemented with penicillin (200 units/ml), streptomycin (200 units/ml), glutamine (4 mM) and 10% (v/v) FCS. A549 were grown in F12 Kaign's medium (F12K), supplemented with penicillin (200 units/ml), streptomycin (200 units/ml), glutamine (4 mM) and 10% (v/v) FCS. HFL-1, pHALFs, MLF and HepG2s were incubated in a humidified atmosphere containing 10% CO<sub>2</sub>. A549 and BEAS-2Bs were maintained in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were routinely tested for mycoplasma contamination using a commercially-available assay (MycoAlert; Cambrex, UK).

Upon reaching visual confluence (3-4 days), cells were sub-cultured (passaged) into new culture flasks. The medium was removed and the cell layer was washed twice with 10 ml Ca<sup>2+</sup>/Mg<sup>2+</sup>-free phosphate buffered saline (PBS) to remove any remaining culture medium. Cells were brought into suspension by adding 1 ml of trypsin (0.05%, w/v)/EDTA (0.02%, w/v) solution and incubating the cells at 37°C until cell detachment occurred. Trypsin was neutralized by addition of 9 ml of the appropriate culture medium supplemented with 10% FCS. Cell suspensions were split at a ratio 1 to 6. Under these conditions cells remained viable for about 10 to 20 passages for HFL-1, A549, BEAS-2Bs and MLFs, and up to 6 to 10 passages for pHALFs.

## 2.17. Preparation of agonists and antagonists

FXa, thrombin, TGF- $\beta_1$ , TNF- $\alpha$ , TLLR, FTLLR, and SLIGKV were reconstituted in DMEM, aliquoted and stored until use at -80°C at the following stock concentrations: FXa (1  $\mu$ M), thrombin (2  $\mu$ M), TLLR, FTLLR and SLIGKV all at 10 mM, TGF- $\beta_1$  (1 ng/ml), and TNF- $\alpha$  (1  $\mu$ g/ml). Prior to use an aliquot of the relevant agonist was defrosted and diluted in the appropriate serum-free medium.

rHir, and ASN were prepared in DMEM prior to aliquoting and freezing at -80°C. The LSKL and SLLK were provided in PBS solution due to their high hydrophilic nature which would have otherwise caused the powder to crystallise during transport. Pertussis toxin was dissolved in tissue culture-tested water. ZK 807834 was dissolved

in acidified saline. All other antagonists and inhibitors were dissolved sterile tissue culture-tested DMSO (*Invitrogen, UK*).

Prior to use, *in vitro* inhibitors were evaluated individually over a range of dilutions in the relevant assay. The maximal working concentrations were determined to obtain optimal neutralisation at doses that do not interfere with basal cell functions. or experiments using rHir and ASN, FXa or thrombin were preincubated with inhibitors in molar excess in serum-free DMEM for 3 hours at 37 °C, with regular shaking, prior to the addition to cell cultures. Control media without inhibitors were incubated under the same conditions.

For experiments involving RWJ-58259 and Ro-31-8425, these compounds were pre-incubated for 20 minutes with cells in fresh medium, and the concentration of DMSO vehicle was standardised amongst all samples. Subsequently thrombin or FXa of the required concentrations were spiked in.

ZK 807834 was preincubated with FXa in serum-free DMEM for 1 hour at 37 °C, with regular shaking, prior to the addition to cell cultures. Control media without inhibitors were incubated under the same conditions.

## **2.18. Cell preparation for experiments**

A suspension of cells trypsinised from one tissue culture flask were placed in a 50 ml sterile polypropylene centrifuge tube. Cells were centrifuged at 1000 rpm for 10min at room temperature using a bench centrifuge (*MSE Mistral 3000, UK*). The supernatant was discarded and the cell pellet was brought into a single cell suspension with 10ml DMEM/10% FCS by gentle mixing. An aliquot of 10 µl of the suspension was removed with a sterile pipette and cells were counted using an improved Neubauer haemocytometer (*BDH-Merck Ltd, UK*). For each experiment, appropriate cell density of the cell suspension was then adjusted with DMEM/10% NCS (see relevant experimental section for cell concentrations).

## **2.19. Western blot analysis of $\alpha$ -SMA, pSMAD/SMAD, TSP-1 and ERK2**

### **2.19.1. Cell culture conditions and sample collection**

pHALFs were prepared as described in **Section 2.18.** and seeded in 12-well plates at a density of  $150 \times 10^3$  cells/well. Cells were allowed to adhere for 24 hours and

subsequently starved in serum-free conditions for a further 24 hours. At the start of the experiment, the medium was removed and replaced with fresh serum-free medium with or without mediators and/or inhibitors as described in **Section 2.17**. After the treatment period, plates were placed on ice, and cells were washed twice with cold PBS. Cells were lysed in 100 µl RIPA buffer (1% PBS, 1% Igepal Ca-630, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulphate) supplemented with complete protease inhibitor cocktail (Complete-mini; *Roche, UK*) and left to stand for 5 minutes. The cell layer was subsequently scraped with a yellow pipette tip and the lysate was transferred into a 1.5ml eppendorf tube. To remove any insoluble cell debris, lysates were spun down at 13,000 rpm for 5 min at 4 °C in a microfuge and the supernatant was transferred into a clean tube. DNA was sheered by passing the sample 15 times through a 25G needle on a 1 ml syringe. Prior to storing samples -80 °C, the protein concentration of each lysate was determined (see **Section 2.19.2**.)

### **2.19.2. BCA protein assay**

Protein concentration of cell or tissue lysates was assessed using the bicinchoninic acid (BCA) protein assay (*Pierce, USA*), as per the manufacturers' protocols. The BCA assay works by the biuret reaction whereby protein reduces  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$  in an alkaline medium. Each  $\text{Cu}^{1+}$  ion then complexes with 2 molecules of bicinchoninic acid (BCA) to form a water-soluble purple-coloured reaction product that exhibits a strong absorbance at 562nm which is linear with increasing protein concentration over a broad working range from 20 µg/ml to 2000 µg/ml. The amount of protein in samples can be derived by comparison a bovine serum albumin standard curve. The assay was performed by adding 200 µl of freshly prepared BCA working reagent to 20 µl of each sample or standard (assayed in duplicate) in a 96-well plate. The plate was agitated on a plate shaker for 30 seconds, and then incubated at 37 °C for 30 min prior to reading the absorbance at 550nm on a Titertek Multiscan MCC/340 plate reader (*Labsystems, Finland*).

### **2.19.3. Western blotting procedures**

Samples were adjusted with RIPA buffer to standardise their total protein concentration. An aliquot of each sample (equivalent to 5 µg of protein for the detection of  $\alpha$ -SMA, 30 µg for detection of pSMAD and TSP-1) was mixed with 5x Laemmli Buffer (3.125 mM TRIS HCL pH 6.8, 10% SDS, 20% Glycerol, 50mM DTT, bromophenol blue (added as required), 4.375 ml  $\text{H}_2\text{O}$ ), boiled for 10 min at 100 °C, then placed on ice prior to loading. Either 30µl of sample or 5µl of SeeBlue® Plus 2 protein ladder (*Invitrogen*) was then added to each well of a 10% SDS-polyacrylamide

gel (made using buffers from National Diagnostics, UK, in a Novex mini-gel system, Invitrogen, UK), and run at 100V in Tris/Glycine/SDS running buffer (0.25M TRIS base, 1.92 M Glycine, 1%SDS).

Following separation, the gel was removed from the cassette and proteins were transferred onto a Hybond-ECL nitrocellulose membrane (*Amersham, UK*), using a horizontal semi-dry transfer method (Multiphor II; *Pharmacia LKB, Sweden*) according to the manufacturer's instructions (transfer buffer was 25 mM Tris-HCl, 0.2 M glycine, 20% methanol), at a current of 1mA per cm<sup>2</sup> of gel, for 1 hour). The quality of protein transfer was assessed by briefly staining the membrane with 2% Ponceau Red solution (*Sigma*) which was destained by a quick wash in double distilled water followed by one wash in tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4;TBS) containing 0.1% Tween20 (TBST). Blots were stored submerged in TBST at 4 °C before enhanced chemiluminescent detection.

#### **2.19.4 Protein Detection**

Blots were incubated with blocking buffer containing 5% nonfat dry milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 hour. Blots were then incubated with anti- $\alpha$ -SMA, anti-phosphoSMAD (pSMAD) or anti-TSP1 antibodies at 1:1000 dilution in 5% skimmed milk in TBST overnight at 4 °C. All blots were then washed 3 x 5 mins in TBST and incubated for 1 hour at room temperature with HRP-conjugated secondary antibody. After further washing in TBST, excess wash solution was drained off, and 2ml of ECL reagent (*Amersham, UK*) was applied to the membrane and incubated for 1 minute. Excess reagent was drained off and immunoreactive bands were visualized by exposing the membrane to autoradiography film (*Kodak, UK*), for between 30 seconds and 10 minutes.

For examination of total ERK2 and Smad2 levels, the blots were washed in TBST and incubated with stripping buffer (62.5 mM 1MTris-HCl (pH 6.8), 2% SDS, 0.1M  $\beta$ -Mercaptoethanol) for 40min at 60 °C. Immunoblotting using anti-total ERK2 or anti-total SMAD2 antibodies was then performed as described above.

#### **2.19.5. Quantification of western blot**

Semi-quantitative analysis of western blots was performed using densitometry. Briefly, blots underwent transmissive greyscale scanning at 300dpi on a standard flatbed scanner (Epson, UK). The scanned images were transferred to the public domain NIH Image 1.61 program (developed at the U.S. National Institutes of Health and available

on the Internet at <http://rsb.info.nih.gov/nih-image/>) and the optical density of each protein band was calculated with reference to a calibration curve (specifically generated by scanning a Kodak Photographic Step Tablet (Kodak, UK) with known optical density gradient, using the same settings as described above). The optical density of the band for the target gene was then normalised against the optical density for the housekeeping gene (ERK2 or SMAD2, as appropriate), allowing correction for protein loading and therefore meaningful comparison between samples.

## 2.20. Immunocytochemical visualisation of actin fibres

pHALFs were seeded in 8-well chamber slides (*Permanox, Nunclon, Fisher Scientific, UK*), and left overnight to adhere in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The medium was then removed, and replaced with serum-free medium for a further 24 hours. This medium was then aspirated, and cells were exposed to FXa (10nM) in 200µl serum-free DMEM for various time points. Cells were then washed three times in PBS, and then fixed and permeabilised in ice cold methanol for 45 seconds, before being washed again in PBS at 4°C.

Fixed cells were incubated for 5 minutes at room temperature with normal goat serum (*Dako, UK*) at a dilution of 1:20 in 200µl PBS, to block non-specific binding sites, then were washed three times with PBS before incubation with mouse anti-human α-SMA antibody, at a dilution of 1:100 in 200µl PBS at for 60 minutes. After a further PBS wash, cells were incubated for 30 minutes with a 1:50 dilution of goat anti-mouse-FITC conjugated antibody (*Dako, UK*) in PBS at room temp in the dark. This solution was then aspirated, and the side-walls of the chambers were carefully removed. Cells were washed a further 3 times in PBS. Coverslips were mounted onto each slide using 3 drops of vectorshield mounting medium (*Vector Laboratories, UK*) containing 4'6-Diamidino-2-phenylindole (DAPI) which is a highly sensitive nucleic acid stain. Images were captured using an inverted fluorescence microscope (*Zeiss Axioskop 2*) with an attached camera (*Olympus C-35DA-2*).

## 2.21. Measurement of TGF-β activity with the tMLEC assay

### 2.21.1. Principles of assay

TGF-β activity in cell culture supernatants, or in co-culture, was measured using a mink lung epithelial cell bioassay. This assay was originally set up by Abe *et al* (Abe *et al*, 1994) in 1994, and utilizes the ability of TGF-β to specifically induce PAI-1

expression. Mink lung epithelial cells were stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene. Exposure of these cells to TGF- $\beta$  results in a dose-dependent increase in luciferase activity in cell lysates. Importantly, this promoter fragment was shown to be only minimally influenced by other known inducers of PAI-1 expression such as bFGF, PDGF-BB, rIL-1 $\beta$ , and EGF (Abe et al, 1994). Hence the assay is highly specific for TGF- $\beta$  activity.

### **2.21.2. Co-cultures**

pHALF and tMLEC cells were harvested by trypsinization and mixed at a ratio of 1:1 and seeded at  $40 \times 10^3$  cells/ml in DMEM/10% FCS in 48-well plates and allowed 24 hours incubation for adhesion and cell spreading. This medium was then changed to serum-free DMEM overnight and medium containing TFLLR or FTLLR (200  $\mu$ M each) supplemented with either 1D11 (concentration), LSKL or SLLK (10 $\mu$ M) was added. The co-culture proceeded overnight, and cells were washed once in PBS before cell lysis and determination of Luciferase activity as described above.

### **2.21.3. Cell culture supernatants**

The assay was performed by plating stably transfected mink lung epithelial cells (tMLEC) at a density of  $1.6 \times 10^4$  cells/well in DMEM/10% FCS in a 96-well tissue culture plate. After allowing cells to adhere for 3 hours the medium was aspirated and cells were washed in serum-free medium. 100 $\mu$ l recombinant porcine TGF- $\beta_1$  standards (0.1-1ng/ml in the same culture medium as the samples) or samples (undiluted medium taken from pHALFs stimulated with or without FXa for 24 hours) were added in duplicate to the plated tMLEC. The plate was then incubated for 14 hours at 37°C/5% CO<sub>2</sub>. Following incubation, media were aspirated and cells were washed in PBS. Subsequently 100 $\mu$ l of luciferase reporter gene assay lysis buffer (Roche, UK) was added to each well, and the plate agitated for 25 minutes. 20 $\mu$ l of each lysate was then transferred into the corresponding well of a 96-well opaque white optiplate (Nunc, UK). The luciferase substrate (Promega, UK), was automatically added and a luminescence reading obtained by the Tropix TR717 Microplate Luminometer (Applied Biosystems, UK).



## **2.22. Methylene blue proliferation study**

### **2.22.1. Principle of the assay**

An assay based on the uptake and release of methylene blue was used to investigate fibroblast proliferation. Methylene blue possesses a positive charge at pH 8.5, and thus binds electrostatically to negatively charged groups, including nucleic acids with phosphate moieties and negatively charged amino acids/proteins within the cell layer. The dye can be eluted from the cell layer by lowering the pH to below 2 with hydrochloric acid. This results in the protonation of acidic groups and liberation of methylene blue into the eluent. Methylene blue forms dimers in aqueous solution at low pH and does not obey Beer-Lambert's Law. This is suppressed by using a hydrochloric acid/ethanol eluent, resulting in a single absorbance peak at 650nm.

### **2.22.2. Cell culture conditions**

All proliferation experiments were performed on pHALFs in sub-confluent culture in the absence of serum. Cells were brought into suspension as described in **Section 2.18**, and seeded into 96-well plates at a concentration of  $5 \times 10^3$  cells in 200  $\mu$ l DMEM. To avoid edge effects, cells were only seeded into the central 10x6 wells of the 96-well plates. The outer wells were filled with 200 $\mu$ l DMEM only. Cells were allowed to attach to the plate over a period of 24 hours after which medium was removed by gentle aspiration and replaced with fresh serum-free medium for a further 24 hours. Subsequently cells were exposed to FXa (25nM) alone or to FXa previously pre-incubated with ZK 807834 for 1 hour in serum-free DMEM (n=6 for each dose for all experiments). The cells were incubated at 37 °C for 48 hours. In order to determine the absorbance of cultures at the onset of each experiment ( $t_0$  absorbance), a plate containing cells plated as described above and left to incubate for the initial 24 hours only, was processed as described in the following paragraph, **Section 2.22.3**.

### **2.22.3. Methylene Blue Assay Procedure**

At the end of the incubation period, the culture medium was removed by blotting the plate over absorbent paper and gentle dipping the plate in a beaker of PBS to wash. The plate was reblotted and the cell layer fixed by addition of 100 $\mu$ l formal saline (10% v/v formalin in 0.15M NaCl) overnight. The plate was wrapped in foil to avoid evaporation of fixative during this period. Formal saline was then flicked off, the plate blotted dry and 100 $\mu$ l freshly filtered methylene blue (1% w/v in 0.01M borate buffer, pH 8.5) was added for 30 minutes. Excess dye was flicked off the plate and the cell layer washed by serially dipping the plate in 4 reservoirs containing 0.01M borate buffer (pH 8.5). After the last rinse, the plate was blotted dry and bound dye was eluted from the

cell layer by addition of 100 $\mu$ l acidified alcohol (0.1M HCL in 50% ethanol). The plate was gently shaken and absorbance for each well measured at 650nm on a platereader (Titertek Multiscan MCC/340 Mk II, *Flow Laboratories, UK*). The platereader was blanked on the first column of wells containing the elution solvent only and results were expressed as fold increase in absorbance/well.

### **2.23. Statistical analysis and expression of data**

All data in figures are presented as mean fold increase  $\pm$  standard deviation unless indicated otherwise. Statistical comparison was performed between two treatment groups by student's t test, and between multiple treatment groups by one way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc testing, using Sigmastat software (*SYSTAT Software Inc, IL, USA*). A *p* value of less than 0.05 was considered significant and individual *p* values for all figures are shown in the relevant figure legends unless value is <0.001.

## Chapter 3: Results

### Overview

The experimental results of this thesis have been divided into three sections. The first section establishes the local production of FX/FXa in the normal and fibrotic mouse and human lung. The second section examines the effect of FXa on fibroblast to myofibroblast differentiation. The third and final section examines the effect of the direct FXa inhibitor ZK 807834 on FXa-induced fibroblast function *in vitro* and in the bleomycin mouse model of lung injury and fibrosis.

### 3.1. Local generation of coagulation zymogen FX in the lung

#### 3.1.1. Introduction

It is generally held that coagulation proteinases such as FX are synthesised in the liver and circulate in the blood as inactive zymogens. In a recent study performed in our laboratory by Drs Scotton and Lee global expression profiling was used during the fibroproliferative response to bleomycin injury in mice to gain novel insights into the mechanism by which the coagulation pathway drives the fibrotic response to lung injury. This study revealed the regulation of 399 non-redundant genes 14 days after bleomycin challenge in C57Bl/6J mice. Many of the regulated genes had previously been identified from microarray analysis of the bleomycin model (Lemay and Haston, 2005; Haston et al, 2005), including collagen I, fibronectin, MMP-12 and PDGF. Surprisingly however, the most highly enriched cluster also contained the gene for the zymogen FX, suggesting that FX is generated locally in the injured lung.

#### 3.1.2. FX gene expression in bleomycin-induced lung injury and fibrosis

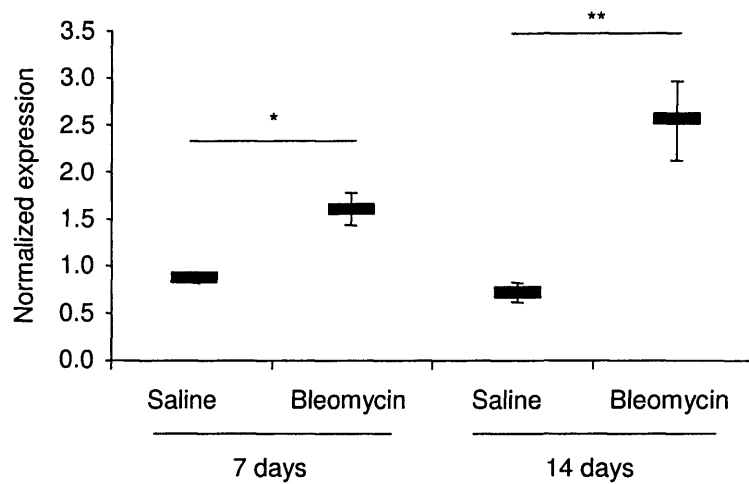
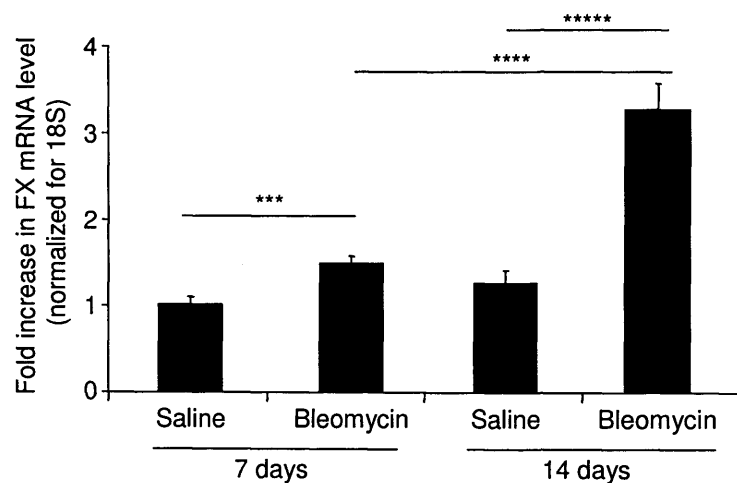
To provide a quantitative and accurate method of validation of the microarray data obtained by Drs Scotton and Lee and to confirm reproducibility of these initial results, FX expression was evaluated in the bleomycin model of lung injury and fibrosis by real-time RT-PCR. Bleomycin was administered by oropharyngeal instillation at a dose of 2mg/kg. This route of administration was chosen preferentially over intratracheal administration which was previously used for all microarray studies to provide a more uniform distribution of pathology within the murine lung (see Discussion **Section 4.1.1.**) Total cellular RNA was prepared from whole lung tissue extracted from mice at 7 and 14 days post-saline or bleomycin challenge. Relative abundance of FX mRNA was established following normalization for the 18S

housekeeping gene. Relative quantitation was calculated according to the  $E^{-\Delta\Delta C_P}$  method.

As demonstrated in **Figure 3.1. (A)** microarray analysis performed by Drs Scotton and Lee demonstrated a significant  $1.61 \pm 0.17$  and  $2.54 \pm 0.73$ -fold increase in FX mRNA levels over saline control at 7 and 14 days respectively. Real time RT-PCR analysis confirmed that the gene for FX was expressed in the murine lung. FX mRNA levels were significantly increased at both 7 and 14 days post-bleomycin injury by 1.62 and 3.57-fold respectively when compared to saline controls (**Figure 3.1. (B)**). These data show that the coagulation zymogen FX is expressed at the mRNA level in the murine lung and that mRNA levels are significantly increased in bleomycin-induced lung injury and fibrosis.

### ***3.1.3. FX gene expression in pulmonary fibrosis induced by aerosolised bleomycin***

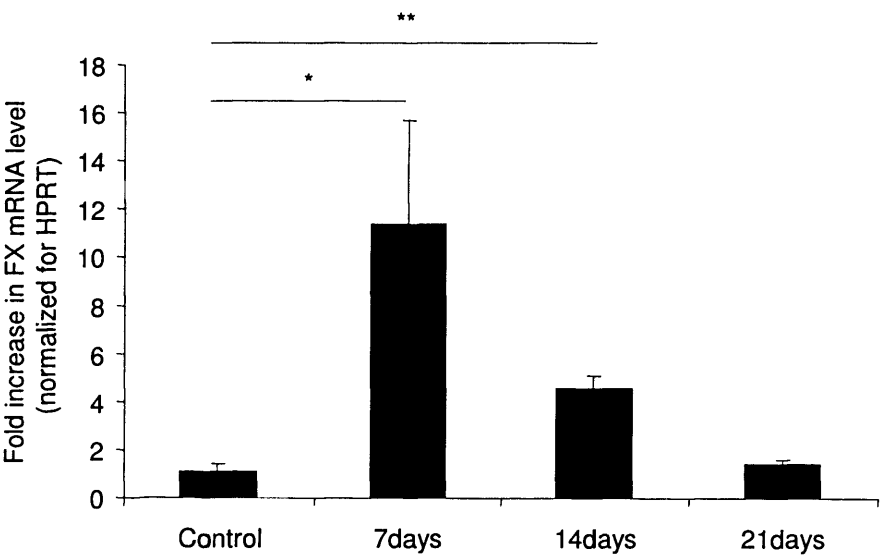
In order to confirm the gene expression of coagulation zymogen FX in bleomycin-induced lung injury and fibrosis, FX mRNA levels were measured in lung tissue obtained from a mouse model of fibrosis induced by aerosolised bleomycin rather than by conventional intratracheal instillation. Animal experiments were performed in Dr Eickelberg's laboratory in Giessen, Germany where mice of similar age to the host laboratories' were orotracheally intubated and mechanically ventilated. Bleomycin (at a dose of 15mg/kg) or saline were administered as an aerosol via a microsyringe (see Methods **Section 2.6.2.**). Lungs were isolated at 7, 14 and 21 days post bleomycin/saline instillation and RNA was analysed from whole lung homogenates by real-time RT-PCR. Relative abundance of FX mRNA was established following normalization for HPRT housekeeping gene. Of note, all real time RT-PCR experiments performed in our laboratory analysed mRNA levels following normalisation to the 18s housekeeping gene. Although this gene proved to be very stable in our samples, this housekeeping gene was unstable in experiments performed in Professor Eickelberg's laboratory, particularly in samples obtained by microdissection. These differences may be explained by the increased susceptibility to degradation of microdissected samples. The HPRT housekeeping gene was therefore chosen for experiments performed in Professor Eickelberg's laboratory.

**A****B**

**Figure 3.1. FX gene expression in bleomycin-induced lung injury and fibrosis.**

(A) Figure shows normalized gene expression from microarray data for murine FX in lung homogenates 7 and 14 days after intratracheal saline or bleomycin. (B) Figure shows FX mRNA levels in mouse lungs assessed by quantitative real-time RT-PCR of whole lung homogenates, collected from mice 7 and 14 days post o.p. challenge with saline or bleomycin (2mg/kg). Data expressed as mean fold increase  $\pm$  s.e.m relative to saline control at 7 days ( $n=3$  in saline group and  $n=5$  in bleomycin group) \*and \*\*and\*\*\*\*and\*\*\*\*\* $p<0.001$ , \*\*\* $p=0.014$

In accordance with the data obtained in our laboratory, FX mRNA was detected at baseline in all samples tested (**Figure 3.2.**). A significant  $11.38 \pm 4.29$ -fold increase in the FX mRNA levels was observed at day 7 post-bleomycin injury and this increase was sustained at day 14 with a  $4.59 \pm 0.47$ -fold increase over saline control.



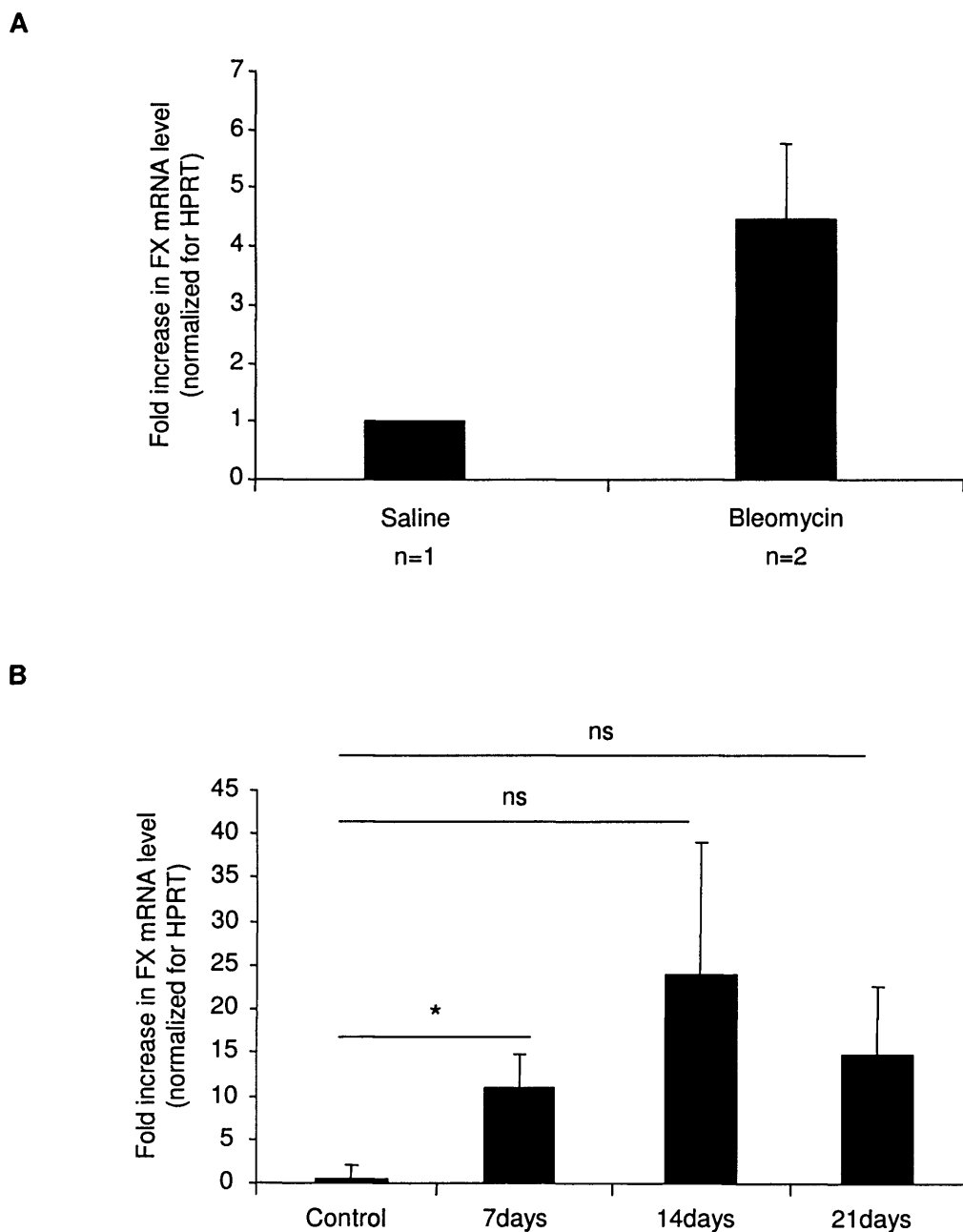
**Figure 3.2. FX mRNA levels in whole lung homogenates at 7, 14 and 21 days post bleomycin- induced lung injury**

Graph shows FX and FVII mRNA levels in mouse lungs assessed by quantitative real-time RT-PCR of whole lung homogenates, collected from mice 7 and 14 days post aerosolised challenge with aerosolised saline or bleomycin (15mg/kg). Data expressed as mean fold increase  $\pm$  s.e.m relative to saline control (n= 3 per group) \*p=0.05, \*\*p=0.014

#### ***3.1.4. FX expression in isolated alveolar epithelial cells post bleomycin-induced lung injury and fibrosis***

To begin to identify the source of FX expression in the murine lung, alveolar epithelial cells and lung fibroblasts isolated from mice challenged with aerosolised bleomycin or saline, were analysed for FX gene expression by real-time RT-PCR. FX mRNA levels were measured for alveolar epithelial cells at the 14 day time point only, as these samples were very difficult to obtain. FX mRNA levels by fibroblasts were measured at 7, 14 and 21 days post-bleomycin challenge. All RNA samples used in this experiment were prepared by Dr Oliver Eickelberg and I performed all cDNA synthesis, primer optimization and real time-RT-PCR experiments in his laboratory. Samples were prepared from cells isolated from whole lung tissue by a dispase digestion technique (see Methods **Section 2.14.** and **2.15.**). Relative abundance of FX mRNA was established following normalization for HPRT housekeeping gene.

As seen in **Figure 3.3.** there was a trend for an increase in FX gene expression in alveolar epithelial cells isolated from bleomycin versus saline-treated lungs, although it was not possible to establish statistical significance, as two replicates in each group had to be excluded reducing the total n number per group to 2 only. These were excluded on the basis of their melting curves which showed multiple peaks indicating the generation of non-specific product. In contrast, a significant  $11.05 \pm 3.81$ -fold increase in FX mRNA levels was observed in fibroblasts 7 days post bleomycin injury compared to saline control and this increase was sustained until 21 days (however, it did not reach significance at both 14 and 21 day time points). These data support the notion that alveolar epithelial cells as well as fibroblasts represent a prominent source of FX production in bleomycin-induced lung injury and fibrosis.



**Figure 3.3. FX gene expression in isolated murine fibroblasts and alveolar epithelial cells post-bleomycin- induced lung injury and fibrosis**

(A) Figure shows FX mRNA levels in alveolar epithelial cells isolated 14 days post aerosolised instillation of aerosolised saline or bleomycin as assessed by quantitative real-time RT-PCR (n=2 per group). (B) Figure shows FX mRNA levels in isolated fibroblast 7, 14, and 21 days post bleomycin and saline challenge. FX mRNA was significantly increased at 7 days with a 11.05-fold increase observed.  $p=0.0058$

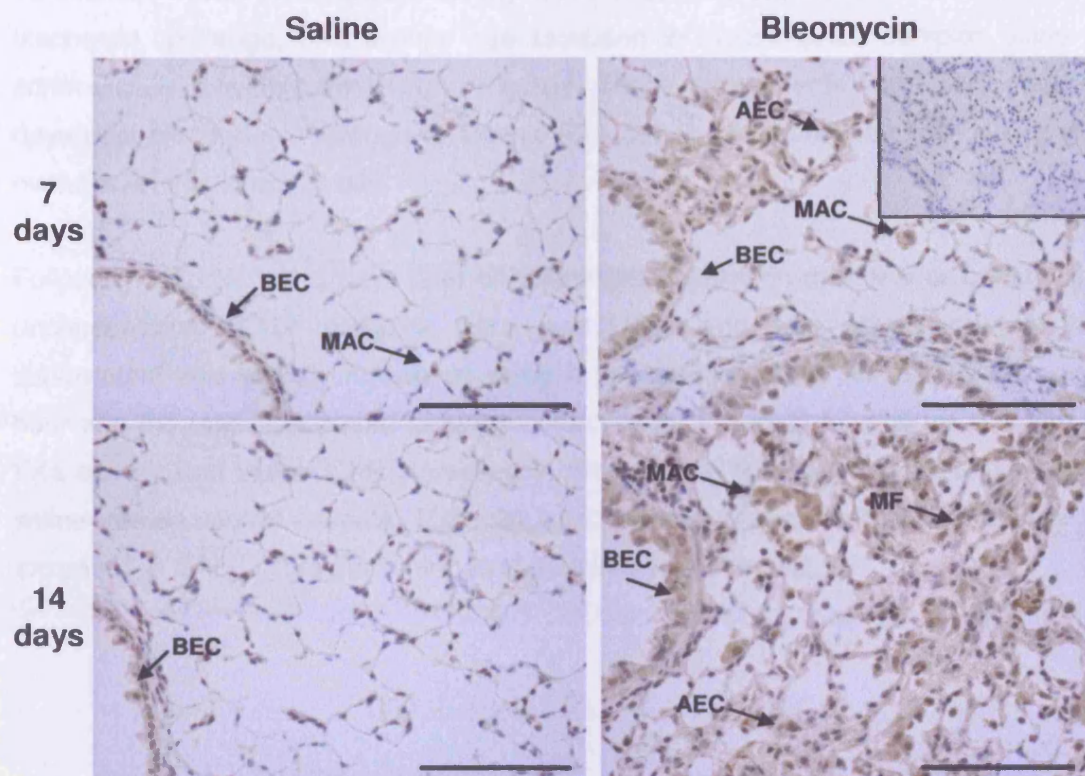


### ***3.1.5. Immunohistochemical localization of FX/FXa protein in bleomycin-induced lung injury and fibrosis***

In order to determine where FX protein localizes in the normal and fibrotic lung, the antibody used for this study recognized both the zymogen and the activated form. FX/FXa protein was examined by immunohistochemistry in lung sections of mice challenged with bleomycin or saline by oropharyngeal instillation for 7 and 14 days.

For optimization of the primary antibody on lung tissue sections, a dilution series was constructed and lung tissue sections were incubated with a polyclonal, goat anti-mouse FX/FXa primary antibody, overnight at 4°C, at varying concentrations (Appendix). Positive staining for FX/FXa was detected by incubating sections with an optimal concentration of the primary antibody (1:200), followed by a biotinylated rabbit anti-goat secondary antibody, a streptavidin/peroxidase complex and a solution of 3,3'-diaminobenzidine (DAB), which generated a brown reaction product. Highly specific staining for FX/FXa was confirmed since control tissue sections incubated with serum only and with an isotype specific, non-immune primary antibody showed no detectable staining. In addition, the antibody specificity was confirmed by western blotting of whole lung and liver homogenates showing single bands at the correct molecular weights for the heavy chain of FX in both the pre-protein and processed conformations.

**Figure 3.4.** shows the immunohistochemical localization of FX/FXa in bleomycin-induced lung injury and fibrosis. Tissue sections from saline-treated animals had very weak brown staining, which was associated with bronchial epithelial cells. However, in the lungs of bleomycin-treated animals 7 and 14 days following instillation, there was intense and widespread brown staining, which was predominantly associated with bronchial and alveolar epithelial cells, but positive signals were also detected with macrophages and myofibroblasts within inflammatory and fibroproliferative foci.



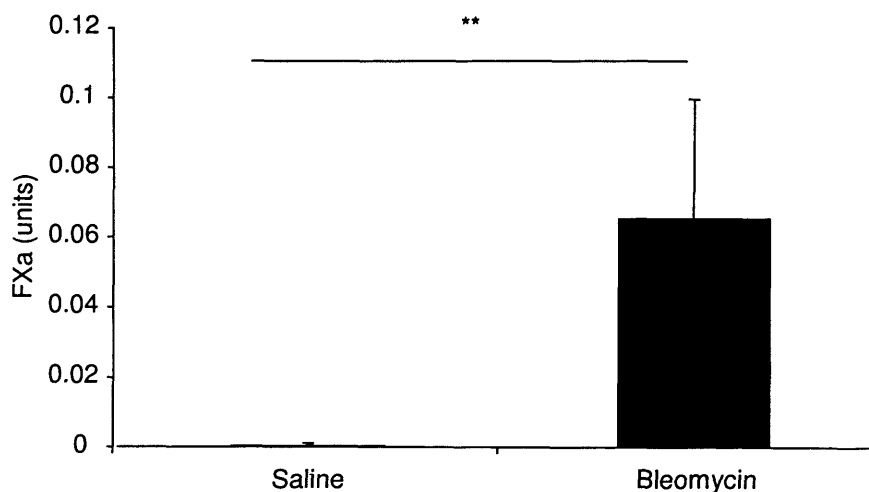
**Figure 3.4. Immunohistochemical localization of FX/FXa in bleomycin-induced lung injury and fibrosis**

Photomicrographs of representative histological sections showing FX/FXa immunoreactivity in mouse lungs 7 and 14 days post o.p. challenge with saline or bleomycin (n=4 per group). There was weak staining for FX/FXa in saline-treated animals at both 7 and 14 days which was restricted to bronchial epithelial cells (**BEC**) and macrophages (**MAC**). Immunostaining was visibly increased in bleomycin-instilled mice at both time points and localised mainly to alveolar (**AEC**) and bronchial epithelial cells as well as macrophages and myofibroblasts in fibrotic foci (**MF**). IgG isotype control section showed no apparent staining (insert shown in the top right hand corner of the 7 days bleomycin section), Specific staining is depicted in brown; nuclei are stained blue with haematoxylin. Scale bars, 100µm.

### 3.1.6. BALF FXa levels in bleomycin-induced lung injury and fibrosis

To examine if local procoagulant activity in the alveolar space was increased following bleomycin challenge, FXa activity was assessed in mouse BALF samples using a commercially available chromogenic assay. These experiments were performed 6 days post-bleomycin challenge to assess FXa activity at a time point preceding the evolution of the fibrotic phase.

Following collection at 6 days post oropharyngeal instillation of saline or bleomycin, unconcentrated BALF samples were centrifuged and light absorbance in the supernatant was serially measured using a spectrophotometer at 405nm over one hour and the rate of thrombin generation calculated. **Figure 3.5.** confirms that BALF FXa activity was significantly increased in mice treated with bleomycin compared to saline-treated control animals ( $150 \pm 26.7$  fold). These data show that FXa activity is increased in BALF in bleomycin-induced lung injury and fibrosis.



**Figure 3.5. FXa levels in BALF following bleomycin instillation**

This Figure shows the effect of oropharyngeal bleomycin on BALF FXa levels after 6 days. FXa activity was determined by chromogenic assay in BALF samples taken at day 6 post intratracheal challenge with 2mg/kg bleomycin or saline. FXa activity increased 150-fold over saline control. Data expressed as mean fold increase  $\pm$  s.d. (n=6 mice per group per group, \*\*p=0.0023).

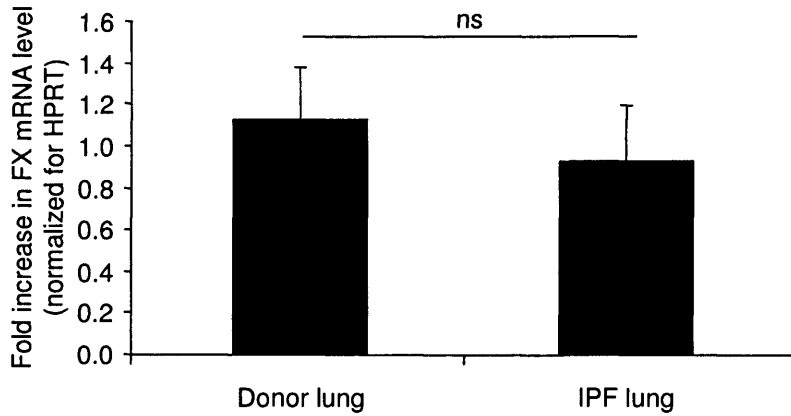
**3.1.7. The zymogen FX is expressed in the human lung and is upregulated in IPF**

Having demonstrated that coagulation FX is expressed locally in the murine lung and is upregulated following bleomycin-induced lung injury and fibrosis, the following set of experiments aimed at examining whether these findings could be translated to human fibrotic lung disease. For these studies mRNA samples extracted from human lung tissue were provided by Dr Oliver Eikelberg, Giessen and all experiments were performed by me in his laboratory.

In the first set of experiments FX mRNA levels were measured by real time RT-PCR in samples taken from total RNA extracted from lung biopsy specimens of patients with UIP or control donor lungs by real-time RT-PCR (**Figure 3.6.**) In all samples tested, FX was clearly detectable but there was no apparent difference between control donor lung and fibrotic lung samples. This is in contrast to the mouse lungs where FX mRNA levels were significantly increased in whole lung homogenates (see **Figure 3.1. (B)**).

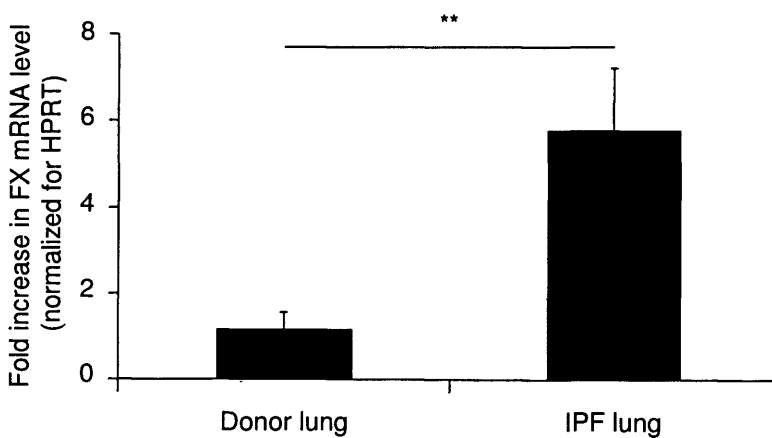
In order to analyze FX gene expression in specific cell populations, subsequent experiments assessed FX expression at the mRNA level in samples obtained from microdissected alveolar septae of patients with UIP compared with the corresponding areas from control lung tissue, whereby alveolar septae are thought to predominantly represent the alveolar epithelium (see **Methods Section 2.13.**). In contrast to data obtained from total lung biopsy material, there was a marked  $5.78 \pm 1.44$ -fold increase in FX mRNA levels for samples obtained from microdissected alveolar septae of patients with UIP compared with the corresponding areas from control lung tissue (**Figure 3.7.**). However, analysis of fibroblasts isolated by trypsin digest from the same patient material showed no significant increase in FX expression in UIP versus donor material, although all fibroblast samples had detectable FX mRNA (**Figure 3.8.**).

These data show that FX is expressed in the human lung and its expression is increased in alveolar epithelial cells from patients with UIP compared with epithelial cells taken from donor lung. Furthermore human lung fibroblasts also express FX at baseline however these do not increase FX gene expression in fibrotic lung disease.



**Figure 3.6. FX mRNA levels in total human fibrotic and donor lung specimens**

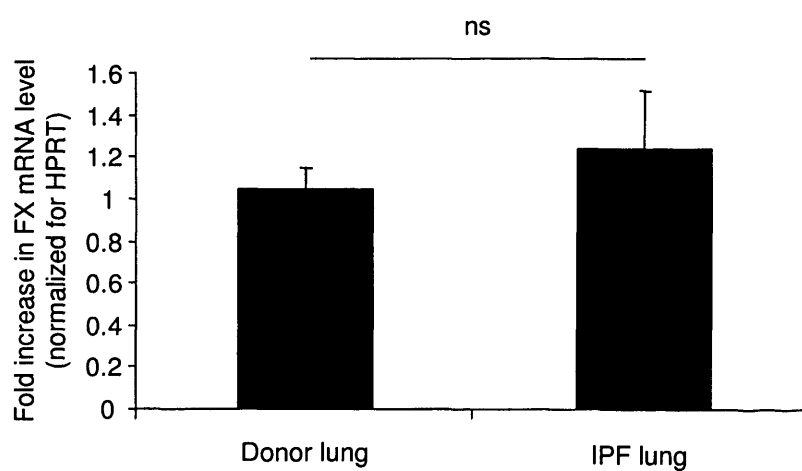
FX expression at the mRNA level as analyzed by quantitative real time RT-PCR. No significant change in FX expression was observed. Data expressed as mean fold increase  $\pm$  s.e.m relative to donor lung in four patients per group.



**Figure 3.7. FX expression in microdissected alveolar septae from human IPF and donor lungs**

This figure shows FX expression at the mRNA level as analyzed by quantitative real time RT-PCR. FX expression is increased  $5.78 \pm 1.44$ -fold in IPF patients compared to control lung. Data expressed as mean fold increase  $\pm$  s.e.m relative to donor lung in four patients per group,

\*\*p=0.005



**Figure 3.8. FX expression in fibroblasts isolated from human donor and fibrotic lung specimens**

FX expression at the mRNA level as analysed by quantitative real time RT-PCR. No significant change in FX expression was observed. Data expressed as mean fold increase  $\pm$  s.e.m relative to donor lung in four patients per group.

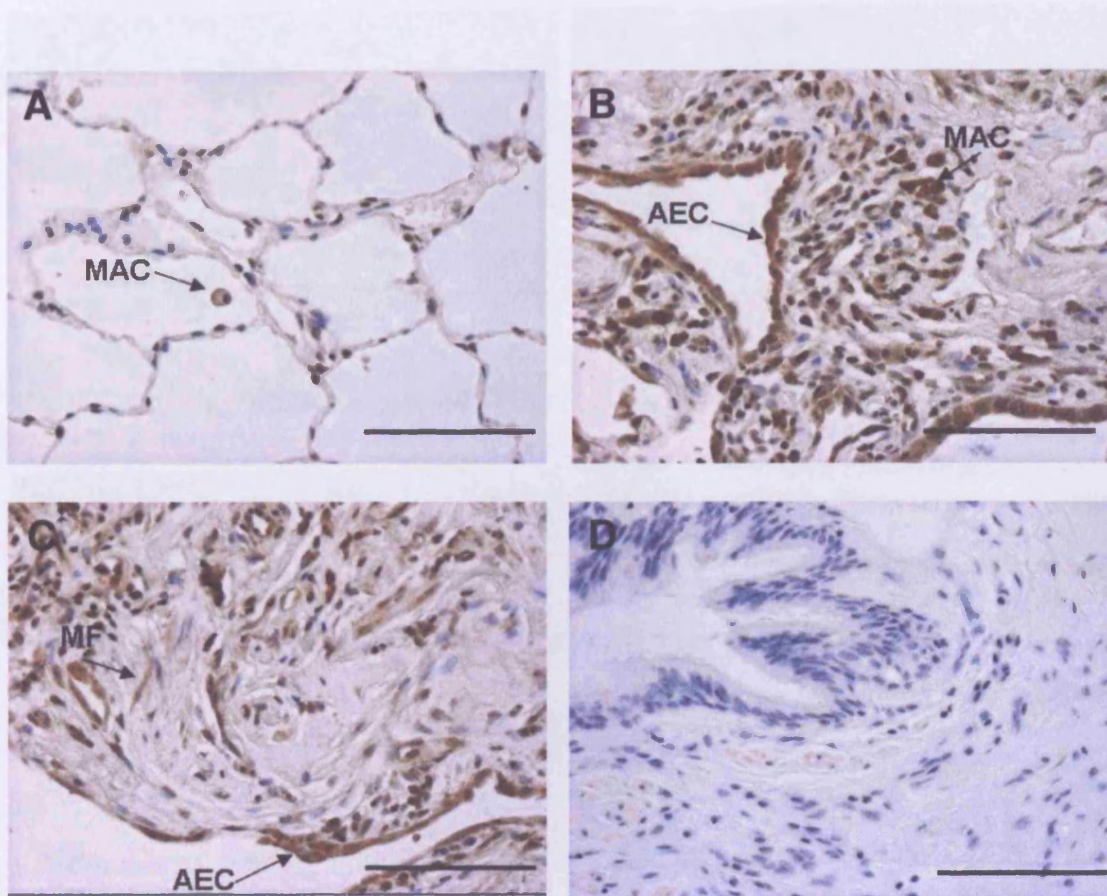
### 3.1.8. Immunohistochemical localization of FX/FXa in IPF

Having confirmed the expression of FX at the mRNA level in the human lung, experiments were performed in order to determine the immunohistochemical localisation of FX and its active form FXa in human donor and fibrotic lung tissues. To this end, human tissue sections taken from 6 donor and 6 IPF lungs were provided by Dr Toby Maher in the host laboratory. Optimisation of the anti-FX/FXa antibody was performed as previously described in **Results Section 3.1.5**.

**Figure 3.9. (A, C, D)** shows photomicrographs of representative histological sections demonstrating immunohistochemical localisation of FX/FXa in human control donor and IPF patients. Control donor lung tissues (n=6) had very weak brown staining, which was occasionally associated with resident macrophages (**Figure 3.9. (A)**). However, in the lungs of 7 patients with IPF there was intense and widespread brown staining confirming strong upregulation of FX/FXa in IPF lungs, which was associated with the bronchial and alveolar epithelium and also with infiltrating macrophages and myofibroblasts within fibrotic foci (**Figure 3.9. (B and C)**). Highly specific staining for FX/FXa was confirmed since control tissue sections incubated with serum only and with an isotype specific, non-immune primary antibody showed no detectable staining (**D**).

This immunostaining pattern was confirmed in human tissue microarrays (TMAs) taken from a second cohort of IPF and control lung biopsy samples provided by Dr Naftali Kaminski (Pittsburgh cohort, n=18; **Figure 3.10.**). TMA are small needle biopsies which allow for detection of 200 or more consecutive section from the same biopsy. The TMAs provided by Dr Kaminski contained 18 small biopsy samples each taken from a different patient with the clinical diagnosis of UIP which were all placed on a single microslide for simultaneous analysis for FX protein expression (see **Methods Section 2.12.3.**). Weak brown staining was again mainly associated with resident macrophages in TMA control donor lung tissues (**Figure 3.10. (A)**). Strong staining for FX/FXa was localised to the bronchial and alveolar epithelium together with macrophages and fibroblasts within fibrotic foci in IPF lung tissue (**Figure 3.19. (B-D)**).

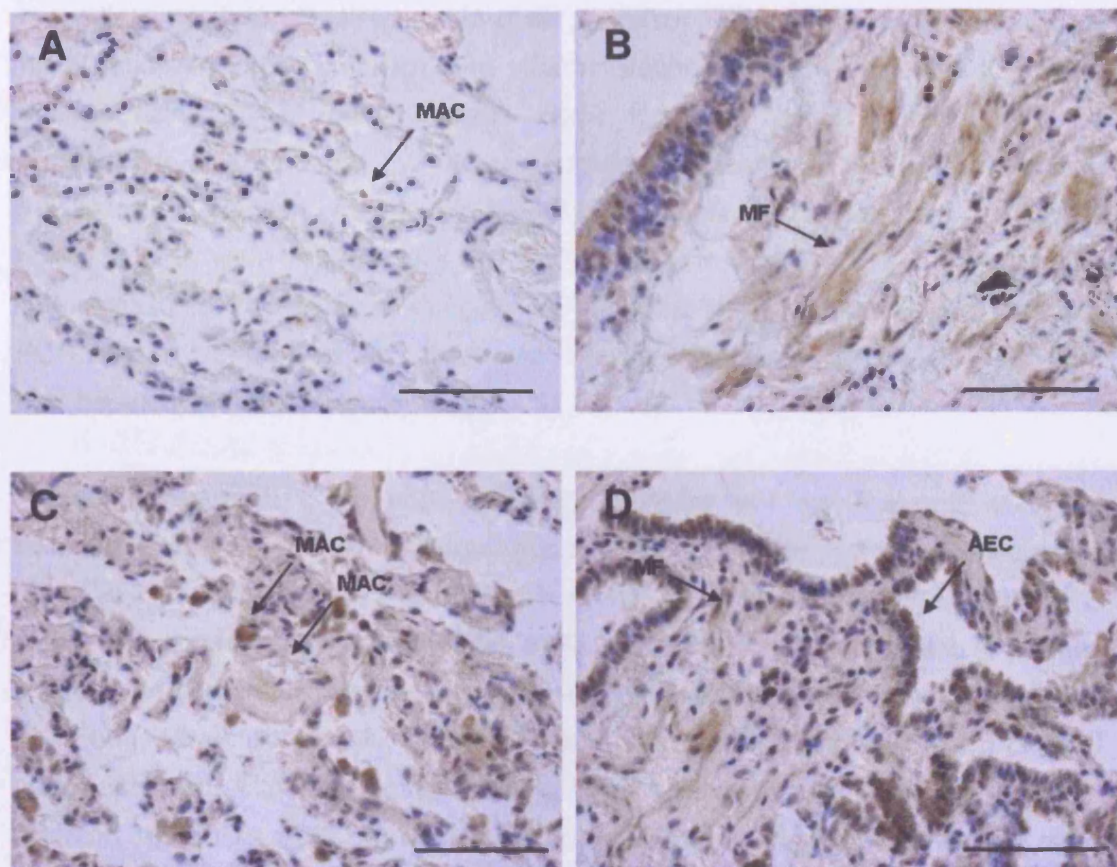




**Figure 3.9. Immunohistochemical localization of FX/FXa in human IPF**

Photomicrographs of representative histological sections showing FX/FXa immunolocalisation in normal human and IPF lung biopsy specimens (Brompton cohort, n=7 in fibrotic lung, n=6 in control group). Specific staining is depicted in brown; nuclei are stained blue with haematoxylin. **(A)** Weak staining was observed in normal control biopsy tissue which mainly localized to alveolar macrophages (**MAC**). **(B and C)** There was marked increase in staining in fibrotic biopsy specimens (two representative sections shown here) which mainly localized to alveolar epithelial cells (**AEC**) but also macrophages and myofibroblasts (**MF**) in fibrotic foci. **(D)** IgG isotype control section showed no apparent staining. Scale bars, 100μm.





**Figure 3.10. Immunohistochemical localization of FX/FXa in human UIP tissue microarrays**

Photomicrographs of representative histological sections showing FX/FXa immunolocalisation in normal human and UIP tissue microarrays (Pittsburgh cohort,  $n=12$  in fibrotic group,  $N=3$  in control group). Specific staining is depicted in brown; nuclei are stained blue with haematoxylin. **(A)** Weak staining was observed in normal control biopsy tissue which mainly localized to alveolar macrophages **(MAC)**. **(B, C and D)** There was marked increase in staining in fibrotic biopsy specimens (representative sections shown here) which mainly localized to alveolar epithelial cells **(AEC)** but also macrophages **(MAC)** and myofibroblasts **(MF)** in fibrotic foci. IgG isotype control shown in **Figure 3.9. (D)**. Scale bars  $100\mu\text{m}$ .

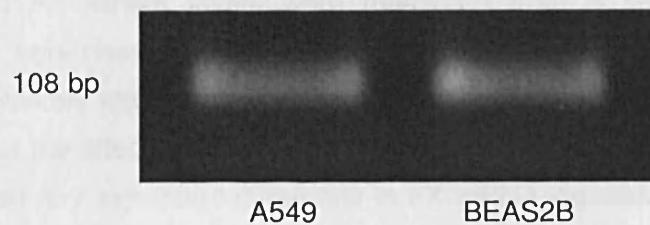
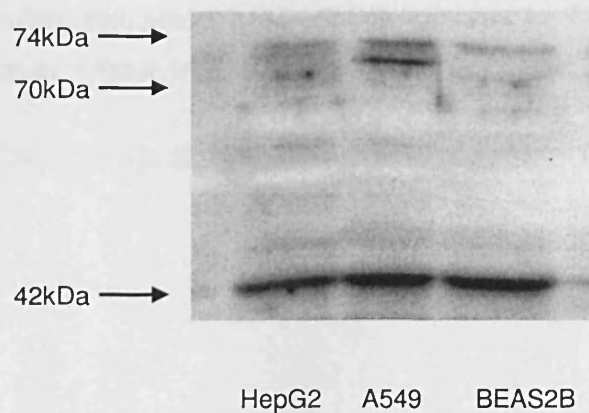
### **3.1.9. FX expression in human alveolar and bronchial epithelial cells in vitro**

In the liver, newly synthesised single-chain precursor FX undergoes post-translational modification, including cleavage and  $\gamma$ -carboxylation which are essential to ensure optimal activation of the zymogen which circulates as a mature two-chain protein (Furie and Furie, 1988; Wallin and Turner, 1990; Stanton and Wallin, 1992) (see **Introduction Section 1.8.1.**). To investigate if cultured alveolar and bronchial epithelial cells were capable of producing the mature form of FX, FX mRNA levels were measured from cultured alveolar and bronchial epithelial cells by real-time RT-PCR and protein derived from these cell lines was compared to that obtained from liver hepatocytes by western blotting.

As seen in **Figure 3.11. (A)**, analysis of RNA extracted from type II alveolar epithelial cells (A549) and bronchial epithelial cells (BEAS-2Bs) confirmed FX expression in both cell lines at the mRNA level. FX protein from A549 and BEAS-2Bs migrated in 3 bands with apparent molecular weights of 74, 70 and 42kD, corresponding to the two single chain precursors and the heavy chain of the mature protein, respectively (Furie and Furie, 1988) (**Figure 3.11. (B)**). FX band pattern observed by western blotting was consistent with the posttranslational processing observed for hepatocytes (Stanton and Wallin, 1992) which are classically viewed as the main cellular source of this zymogen. These data support the notion that alveolar and bronchial epithelial cells produce FX and have the capacity to induce correct processing of the mature FX zymogen.

### **3.1.10. Bleomycin induces FX expression in alveolar epithelial cells**

A series of *in vitro* studies were undertaken in an attempt to identify potential mediators of FX expression in alveolar epithelial cells. To this end the human lung epithelial cell line A549 was grown to 80% confluency in 6-well plates and after 18 hour starvation it was exposed to a variety of different stimuli over time. FX mRNA levels were analysed by real time RT-PCR. Initial experiments examined the effect of TGF- $\beta$  and TNF- $\alpha$  on FX expression by A549. Increased levels of both cytokines have been measured in both animal models of pulmonary fibrosis as well as in human fibrotic lung disease and have been implicated in the pathogenesis and progression of pulmonary fibrosis (see **Introduction Section 1.3.**).

**A****B**

**Figure 3.11. FX expression at the mRNA and protein level in human lung epithelial cells in vitro**

**(A)** Quantitative expression of FX in A549 and BEAS2B at the mRNA level. **(B)** FX protein expression of HepG2, A549 and BEAS2B by western blotting. Results are representative of three separate experiments.

---

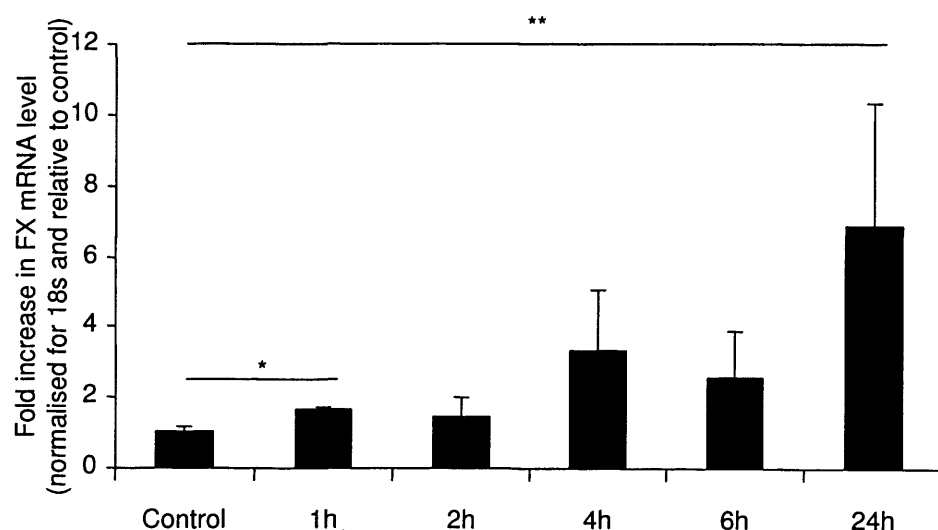
Cells were exposed to a single dose of TGF- $\beta$  (2ng/ml), TNF- $\alpha$  (1ng/ml) in serum-free conditions and FX mRNA levels were measured from 3 hours onwards. These concentrations were chosen based on those described previously for TGF- $\beta$ - and TNF- $\alpha$ -induced stimulatory effects on alveolar epithelial cells (Willis et al, 2005). **Table 3.1.** is a summary of the effects of TNF- $\alpha$  and TGF- $\beta$  on  $\alpha$ -SMA expression. None of these stimuli produced any significant difference in FX mRNA expression at any given time point.

The effect of bleomycin on FX expression in alveolar epithelial cells was also examined. A549 were stimulated with a single concentration of bleomycin (50U/ml) and FX mRNA levels were measured over time by real time RT-PCR. As seen in **Figure 3.12.** bleomycin stimulated a significant increase in FX mRNA levels that reached significance at 1 hour post stimulation and escalated over a 24 hour time period.

Time(hours)	Control	TGF- $\beta$	TNF $\alpha$
3	1.02 $\pm$ 0.27	2.11 $\pm$ 0.09	1.43 $\pm$ 0.01
6	1.46 $\pm$ 0.17	1.71 $\pm$ 0.21	1.63 $\pm$ 0.52
16	2.94 $\pm$ 0.16	1.93 $\pm$ 0.16	1.24 $\pm$ 0.74

**Table 3.1. Effect of TGF- $\beta$  and TNF- $\alpha$ -stimulation on FX mRNA levels in A549**

FX mRNA levels as analyzed by quantitative real time RT-PCR. No significant increase in FX expression was observed at any time point. Data expressed as mean fold increase  $\pm$  s.d, three replicates per group,



**Figure 3.12. Effect of bleomycin stimulation on FX mRNA levels in alveolar epithelial cells**

Fold change (normalized for 18s) in FX mRNA levels was measured by real-time RT-PCR for A549 human alveolar type II cells after 18 hour serum starvation followed by incubation with 50U/ml concentration of bleomycin for 1, 2, 4, 6 and 24 hours. Data represent mean fold change  $\pm$  standard deviation between the samples, n=3 for all samples, representative experiment of three replicates \*p=0.004, \*\*p=0.03.

### 3.1.11. Summary

The results described in this section, examining the local production of FX/FXa in the murine and human lung showed:

- FX is expressed in the murine lung and is upregulated in bleomycin-treated mice compared to saline-treated controls
- FX/FXa protein was localized mainly to alveolar and bronchial epithelial cells but also to fibroblasts and alveolar macrophages in bleomycin-treated animals.
- Alveolar epithelial cells isolated from mouse lungs post-bleomycin injury show a trend for increased expression of FX compared to saline controls.
- Fibroblasts isolated from mouse lungs post-bleomycin injury significantly upregulate FX expression at the mRNA level compared to saline control.
- BALF FXa levels were elevated in bleomycin treated mice compared to saline treated controls.
- Laser-capture microdissection of human alveolar septae but not whole lung homogenates demonstrated marked upregulation of FX gene expression in IPF patients compared with control lung. However, fibroblasts isolated from human donor and IPF lung showed no increase in FX expression at the mRNA level.
- FX/FXa protein localisation showed consistent immunoreactivity of FX/FXa associated with the bronchial and alveolar epithelium, macrophages and fibrotic foci, in biopsy tissue samples from 18 patients with UIP which were derived from independent cohorts in the USA and the UK.
- Alveolar and bronchial epithelial cells in culture express FX at the mRNA and at the protein level. FX protein produced by these cells appears to undergo equivalent processing to that of hepatocytes.
- Bleomycin stimulates an increase in FX gene expression in alveolar epithelial cell *in vitro*.

In summary, the results presented in this thesis show that FX is expressed and upregulated in bleomycin-induced lung injury and fibrosis and IPF, supporting the notion of local production of the coagulation system components in fibrotic lung disease. Furthermore, this thesis identified the alveolar epithelium as a major source of FX expression within the fibrotic lung and further supports the notion that alveolar epithelial cells may produce the mature form of the zymogen.

## **3.2. The role of FXa in fibroblast to myofibroblast differentiation**

### **3.2.1. Introduction**

There is strong evidence that the differentiation of fibroblasts into myofibroblasts plays a central role in the pathogenesis of pulmonary fibrosis, as these cells are highly synthetic in terms of the production of extracellular matrix proteins and appear to be resistant to apoptosis (reviewed in (Phan, 2002)). In the previous results section, the observation was made that fibroblasts/myofibroblasts within fibrotic foci stain positively for FX/FXa by immunohistochemistry in the bleomycin model of lung injury and fibrosis as well as in human fibrotic samples. This led to the hypothesis that fibroblasts are major target cells for FXa in fibrotic lung disease. The following section examines this hypothesis by evaluating the effect of FXa on fibroblast to myofibroblast differentiation *in vitro*.

Myofibroblast differentiation was studied *in vitro* using primary human adult lung fibroblasts (pHALFs) previously isolated in the host laboratory from lung explants (for a more detailed description see Methods **Section 2.15.**). These cells were chosen since they provide the closest representation of human biology, compared to ordinary immortalized cell lines. In addition, they have been well characterized in our laboratory and have previously been used to establish the effects of thrombin and TGF- $\beta$  stimulation on fibroblast function (Chambers et al, 2003). Myofibroblast differentiation was assessed by determining *de novo* expression of  $\alpha$ -SMA, the cytoskeletal protein recognized as the most significant marker of the myofibroblast phenotype (Chaponnier and Gabbiani, 2004).

### **3.2.2. Effect of FXa on primary adult human lung fibroblast to myofibroblast differentiation**

To initially determine the effect of FXa on myofibroblast differentiation, a detailed dose-response experiment was performed. Cells were grown to 80% confluency and exposed to FXa at a range of concentrations (100pM-50nM) in serum-free DMEM. This range of concentrations was chosen based on previous studies conducted in our laboratory (Blanc-Brude et al, 1999).  $\alpha$ -SMA protein levels in whole cell layer extracts were measured by western blotting and the intensity of the bands was analyzed by densitometry (relative to the intensity of the bands for total ERK2 used as loading control).

**Figure 3.13.** shows a representative experiment for the effect of FXa on fibroblast  $\alpha$ -SMA expression. The anti- $\alpha$ -SMA antibody recognized a faint 42-kDa band in unstimulated pHALFs. The intensity of this band was highly increased in cells exposed to FXa. Densitometric analysis of the protein bands revealed that FXa increased fibroblast  $\alpha$ -SMA protein levels in a concentration-dependent fashion between 10nM and 50nM. At 10nM FXa stimulated  $\alpha$ -SMA protein level by  $29.88 \pm 5.6$  -fold relative to DMEM control and this response was maximal at 25nM as  $\alpha$ -SMA protein levels increased by  $57.53 \pm 6.15$ -fold over media control. At 50 nM, which was the highest concentration measured no further increase in  $\alpha$ -SMA protein levels could be observed ( $51.76 \pm 12.61$ -fold). These data show that FXa is a potent stimulus for fibroblast  $\alpha$ -SMA expression.

Based on these results, a dose of 10nM of FXa was chosen for all subsequent experiments. This concentration falls below the physiological concentration of its precursor (140nM; (Laurent and Shapiro, 2007)) and is similar to concentrations of FXa previously described to induce stimulatory effects in fibroblast-, smooth muscle cell-and endothelial cell proliferation studies (Blanc-Brude et al, 2005;Herbert et al, 1998;Nicholson et al, 1996).This concentration is also most likely to mediate a substantial increase in  $\alpha$ -SMA expression with the least off-target effects, which are commonly observed at high concentrations of coagulation proteinases in general (Chambers and Laurent, 2002).

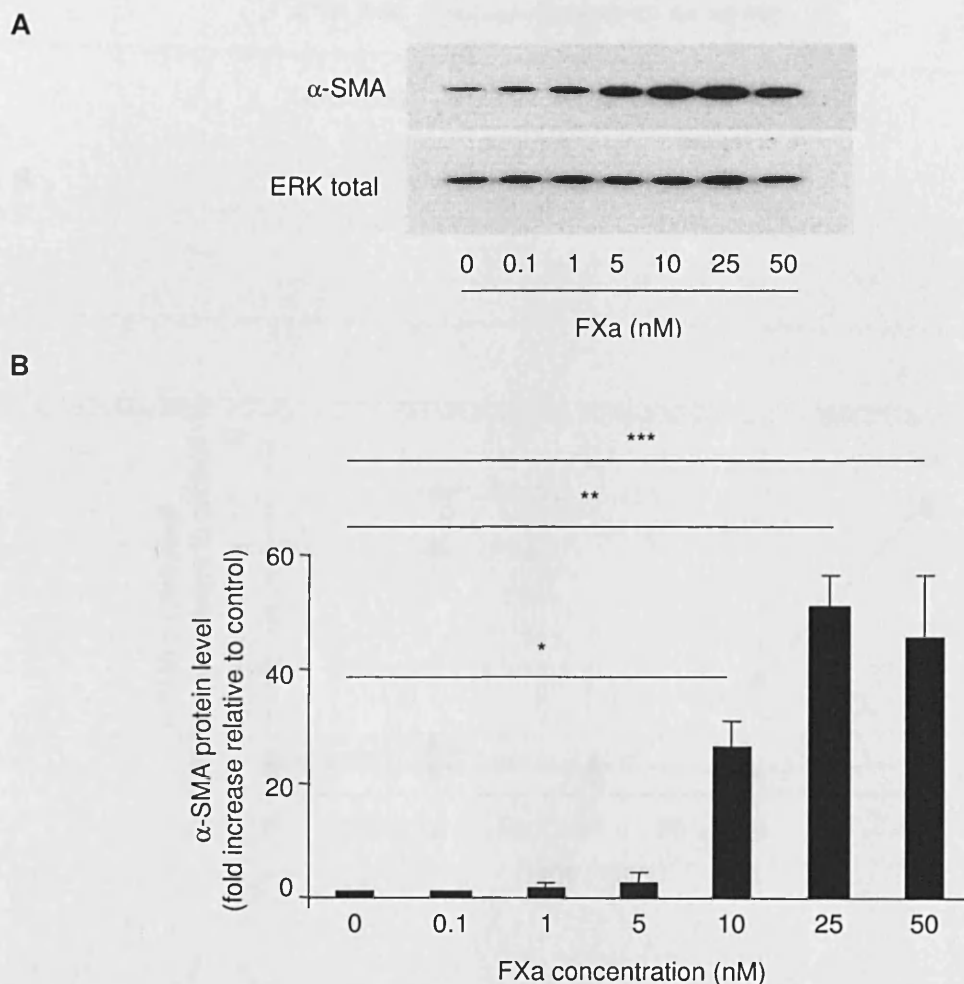
### **3.2.3. Kinetics of myofibroblast differentiation induced by FXa**

The kinetics of the FXa-induced  $\alpha$ -SMA expression were subsequently determined. Cells were exposed to a single dose of FXa (10nM) in serum-free conditions and  $\alpha$ -SMA protein levels were measured by western blotting from 24hours up to 48hours.

As shown in **Figure 3.14.**, the response was not immediate, with the earliest detectable increase in  $\alpha$ -SMA protein levels observed at 36hours ( $3.74 \pm 1.19$ -fold). Control fibroblasts treated with DMEM only, showed no significant increase in  $\alpha$ -SMA protein levels over time. At 48hours, which was the last time point measured, the response was maximal compared to previous time-points with an observed increase of  $9.45 \pm 0.31$ -fold over medium control cells. Similar kinetics in  $\alpha$ -SMA expression were observed in three separate experiments that were performed. These data show that the effect of FXa on  $\alpha$ -SMA expression is time-dependent. Given that 36hours was the first time point at which a significant effect was observed, all subsequent experiments

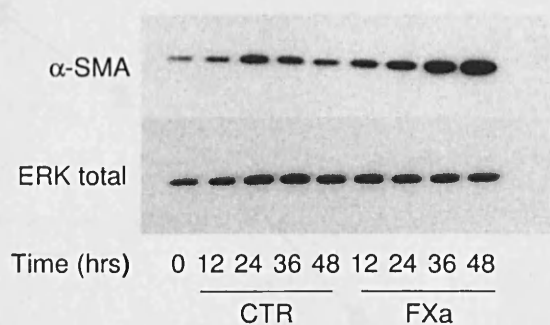
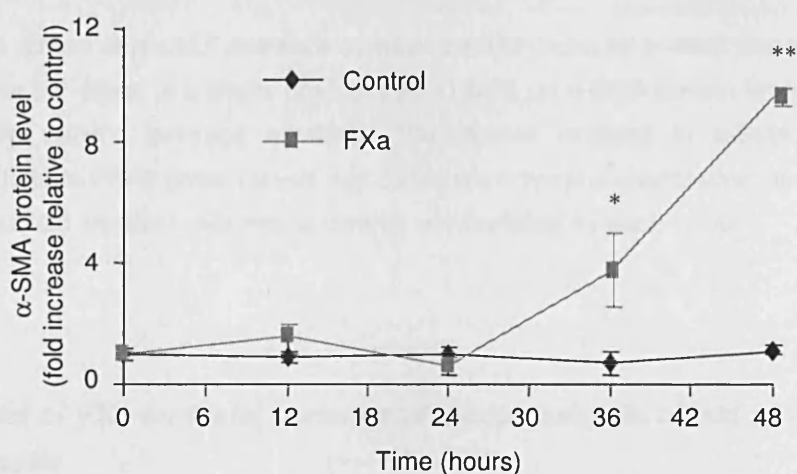


assessing FXa-induced  $\alpha$ -SMA protein levels were performed at this time point. In addition, pHALFs were used at passage 4 to 8 only based on the observation that fold increase in  $\alpha$ -SMA expression in response to FXa rapidly decreased with an increase in passage number (passage 4, 29.88- fold and passage 8, 2.91-fold, **see Figure 3.15.**).



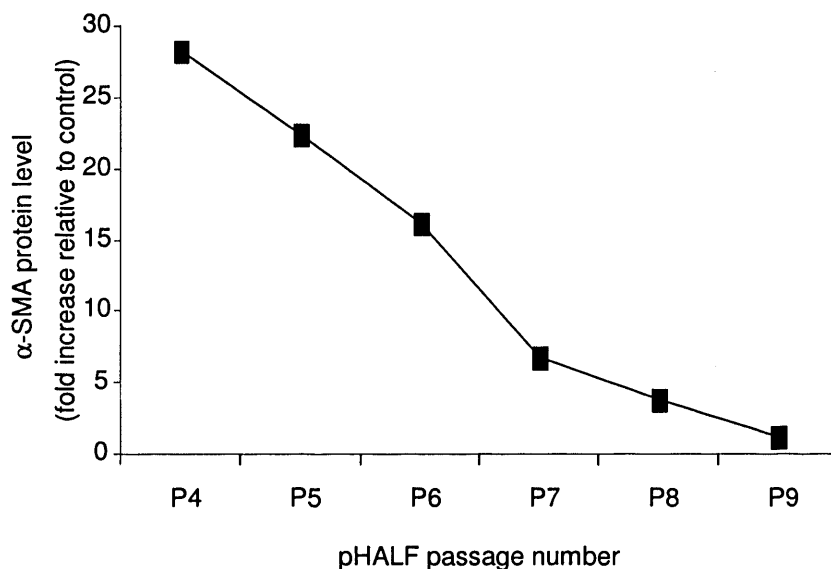
**Figure 3.13. FXa induces fibroblast  $\alpha$ -SMA expression in a concentration-dependent manner.**

(A) Figure shows the effect of varying concentrations of FXa on  $\alpha$ -SMA protein levels.  $\alpha$ -SMA protein was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as the protein loading control. (B) The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Representative experiment of two performed with three replicates for each value expressed in fold increase over control  $\pm$  s.d. \* and \*\* and \*\*\* $p < 0.001$

**A****B**

**Figure 3.14. FXa induces fibroblast to myofibroblast differentiation in a time-dependent manner.**

Figure shows the effect of time on FXa-induced α-SMA protein levels. **(A)** pHALFs were exposed to a single dose of FXa (10nM) and α-SMA protein levels were detected using an anti-α-SMA antibody and blots were then stripped and reprobed with total ERK2 as the protein loading control. **(B)** The relative increase in α-SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Representative experiment of three separate experiments performed with three replicates for each value, data expressed as mean fold change relative to control ± s.d. \*p=0.013, \*\*p<0.001



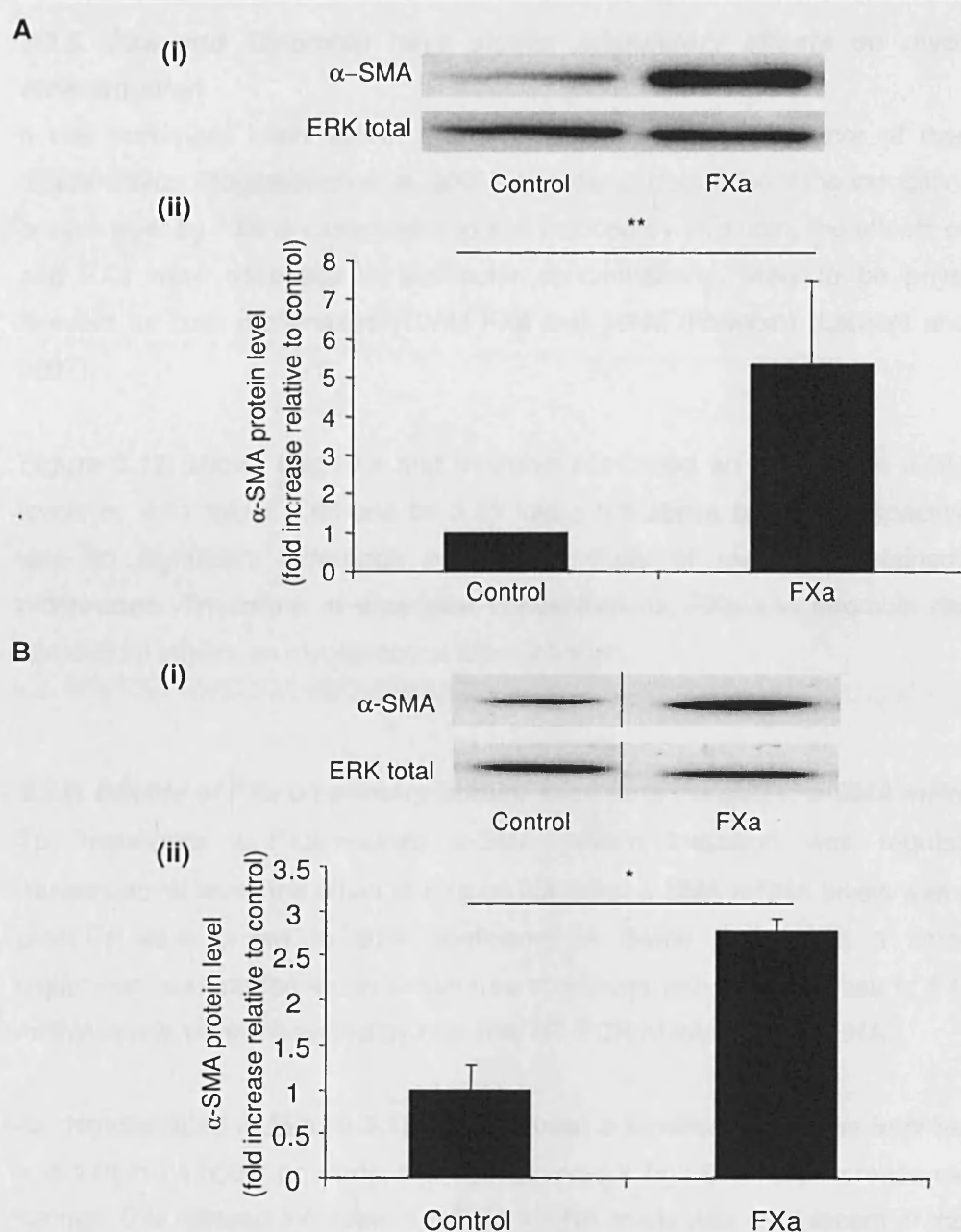
**Figure 3.15. Effect of pHALF passage number on FXa-induced  $\alpha$ -SMA expression.**

Figure shows the effect of a single dose of FXa (10nM) on  $\alpha$ -SMA protein levels at 36hours in pHALFs with varying passage numbers. The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Data expressed as fold increase over media control, one replicate for each value.

#### **3.2.4. Effect of FXa on foetal human and mouse lung fibroblast to myofibroblast differentiation**

To confirm that the effects of FXa on fibroblast to myofibroblast differentiation were not unique to pHALFs, the ability of FXa to induce  $\alpha$ -SMA expression was confirmed in other cell types, including a human foetal lung fibroblast cell line (HFL-1) and murine lung fibroblasts (MLFs). HFL-1 and MLFs were grown in identical conditions to pHALFs and exposed to FXa (10nM) for 36hours.

**Figure 3.16.** shows that FXa is a potent inducer of  $\alpha$ -SMA expression in both HFL-1 and MLFs, with observed increases of 5.33 fold  $\pm$  2.16 and 2.76 fold  $\pm$  0.15 in  $\alpha$ -SMA protein levels over media control respectively. These data further confirm that FXa is a potent inducer of the  $\alpha$ -SMA expression in mouse and human lung fibroblasts.



**Figure 3.16. FXa induces  $\alpha$ -SMA expression in human foetal and mouse lung fibroblasts**

Figure shows the effect of FXa on  $\alpha$ -SMA protein levels in mouse lung fibroblast (**A**) and human foetal (**B**) cell lines. (**A (i) and B (i)**) Cells were exposed to a single dose of FXa (10nM) and  $\alpha$ -SMA protein levels were detected using an anti- $\alpha$ -SMA antibody and blots were stripped and reprobed with total ERK2 as the protein loading control. (**B (i)**) Lines indicate that protein bands were not positioned directly adjacent to each other on the blot. The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by quantitative densitometry (**A (ii) and B (ii)**). Representative experiment for three separate experiments performed. All data represented as mean fold increase over media control  $\pm$  s.d. \*\* and \* $p < 0.001$

### **3.2.5. FXa and Thrombin have similar stimulatory effects on myofibroblast differentiation**

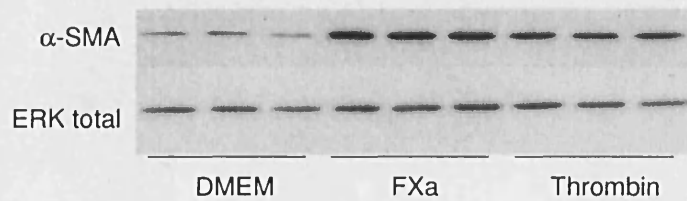
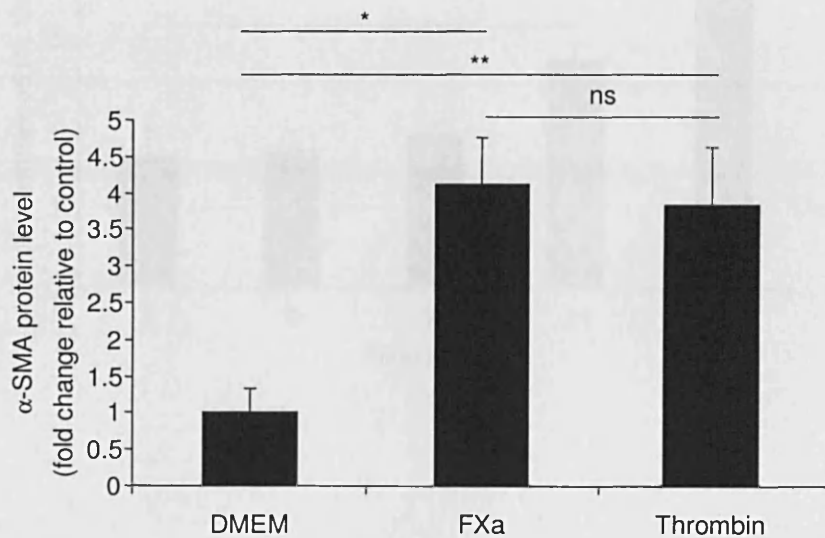
It has previously been shown that thrombin is a potent mediator of myofibroblast differentiation (Bogatkevich et al, 2001). In order to determine if the induction in  $\alpha$ -SMA protein level by FXa is comparable to that induced by thrombin, the effects of thrombin and FXa were assessed at equimolar concentrations, likely to be physiologically relevant for both proteinases (10nM FXa and 10nM thrombin) (Laurent and Shapiro, 2007).

**Figure 3.17.** shows that FXa and thrombin stimulated an increase in  $\alpha$ -SMA protein levels by  $4.11 \pm 0.63$  and by  $3.83 \pm 0.8$  above baseline respectively. There was no significant difference in the magnitude of response obtained for both proteinases. Therefore, at equimolar concentrations, FXa and thrombin have similar stimulatory effects on myofibroblast differentiation.

### **3.2.6. Effects of FXa on primary human adult lung fibroblast $\alpha$ -SMA mRNA levels**

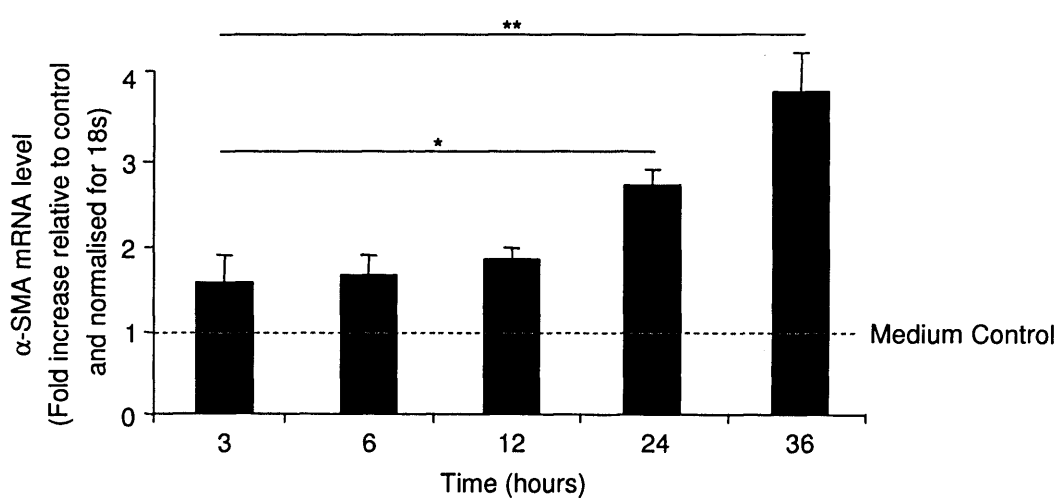
To investigate if FXa-induced  $\alpha$ -SMA protein induction was regulated at a transcriptional level, the effect of FXa on fibroblast  $\alpha$ -SMA mRNA levels was assessed. pHALFs were grown to 80% confluency in 6-well plates and a time-response experiment was performed in serum-free conditions with a single dose of FXa (10nM). mRNA levels were measured by real time RT-PCR of total cellular RNA.

As demonstrated in **Figure 3.18.**, FXa caused a significant increase in  $\alpha$ -SMA mRNA levels from 24 hours onwards, with an observed  $2.74 \pm 0.17$ -fold increase over DMEM control. This delayed increase in  $\alpha$ -SMA mRNA levels was reminiscent of the increase in FXa-induced  $\alpha$ -SMA expression at the protein level (see **Figure 3.1.4.**). The response was maximal compared to other time points measured at 36 hours and showed a  $3.82 \pm 0.47$ -fold increase in the  $\alpha$ -SMA mRNA levels compared to control. Fibroblasts treated with DMEM alone showed no significant difference in  $\alpha$ -SMA mRNA levels over time, suggesting that the increase is specific to FXa stimulation. These findings were confirmed in three separate experiments performed. These data therefore show that FXa transcriptionally regulates  $\alpha$ -SMA expression.

**A****B**

**Figure 3.17. FXa and thrombin have similar effects on α-SMA expression**

Figure shows the effect of thrombin and FXa on α-SMA protein levels. **(A)** pHALFs were exposed to FXa and thrombin at equimolar concentrations (10nM) and α-SMA protein was detected using an anti-α-SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. **(B)** The relative increase in α-SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Representative experiment of three experiments performed with three replicates for each value expressed in fold increase above media control ± s.d. \*p=0.0016 \*\*p=0.0048



**Figure 3.18. Effect of FXa stimulation on  $\alpha$ -SMA gene expression in pHALFs.**

Figure shows the effect of FXa (10nM) stimulation on  $\alpha$ -SMA mRNA levels in pHALFs over time, up to 36hours.  $\alpha$ -SMA mRNA levels were determined by real time RT-PCR. Representative experiment of three separate experiments performed with three replicates per value. mRNA levels expressed as fold increase relative to their respective time-point matched control  $\pm$  s.d, media controls replaced by a single dotted line for the sake of simplicity.

\*and \*\* $p < 0.001$

### 3.2.7. Effect of FXa on $\alpha$ -SMA fibre formation on human adult lung fibroblasts

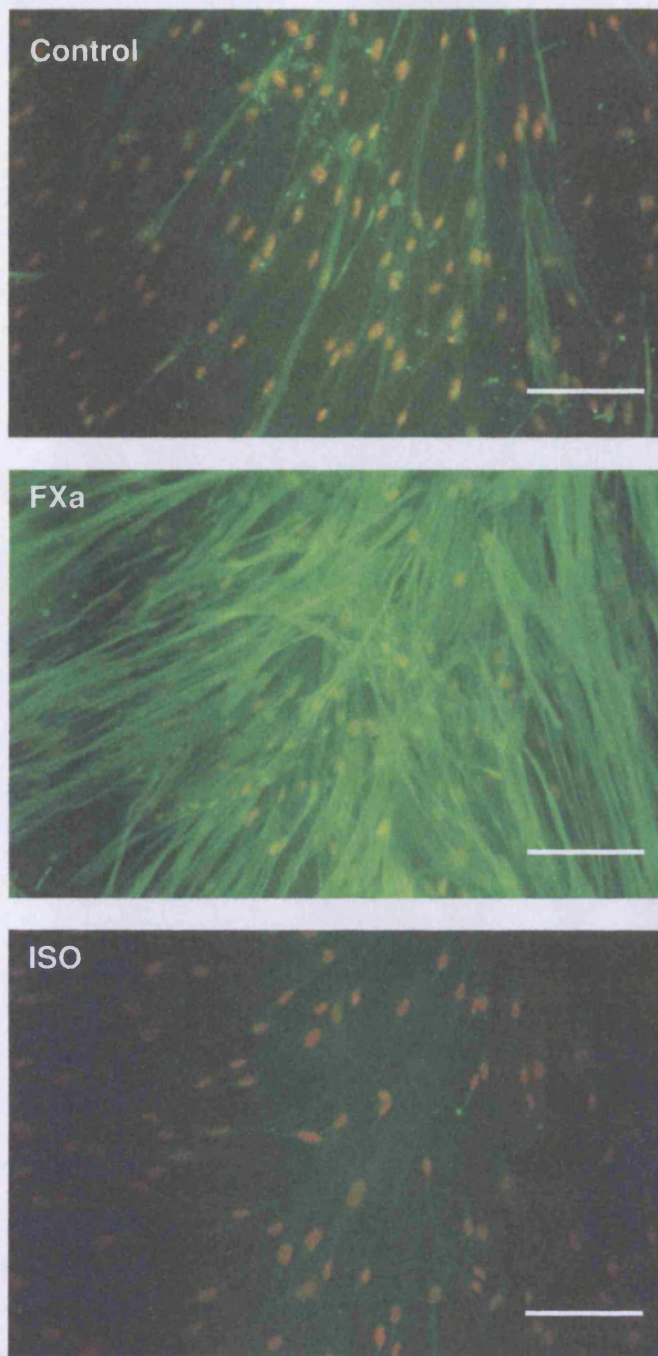
$\alpha$ -SMA is a mechanosensitive protein that in response to mechanical stress assembles into stress fibres which are characteristic of the myofibroblast phenotype (see **Introduction Section 1.5.**). To determine whether FXa induced the assembly of  $\alpha$ -SMA stress fibres,  $\alpha$ -SMA protein was visualized by immunocytochemical analysis. pHALFs were grown to subconfluence in chamber slides, quiesced for 24 hours and exposed to FXa and DMEM for 36 hours prior to methanol fixation. Cells were subsequently incubated with a primary anti- $\alpha$ -SMA antibody or non-specific IgG isotype control antibody and a secondary fluorescein isothiocyanate-conjugated antibody and visualised by immunocytofluorescent microscopy.

**Figure 3.19.** shows representative images of the effect of FXa stimulation on  $\alpha$ -SMA fibre formation. In accordance with data obtained by western blotting, pHALFs expressed low levels of  $\alpha$ -SMA in serum-free conditions which did not appear to assemble into stress fibres. After exposure to FXa for 36 hours, fluorescence for  $\alpha$ -SMA was highly increased and was visible as an extensive network of brightly stained fibres which assembled into stress fibres resembling the stress fibres which are characteristic of the myofibroblast phenotype. This phenotype persisted up to 96 hours which was the last time point investigated (**Figure 3.20.**).

Stress fibre assembly induced by FXa was also confirmed in a second fibroblast line-HFL-1 (**Figure. 3.21.**). Compared to pHALFs, HFL-1 showed an increased baseline expression in  $\alpha$ -SMA protein, which was not assembled into stress fibres. 36 hours post stimulation with FXa (10nM) positive staining was markedly increased and  $\alpha$ -SMA positive fibres formed with a morphology very reminiscent of that observed for pHALFs. Thrombin-induced  $\alpha$ -SMA fibre formation after 36 hours was used as a positive control and showed a very similar staining pattern.

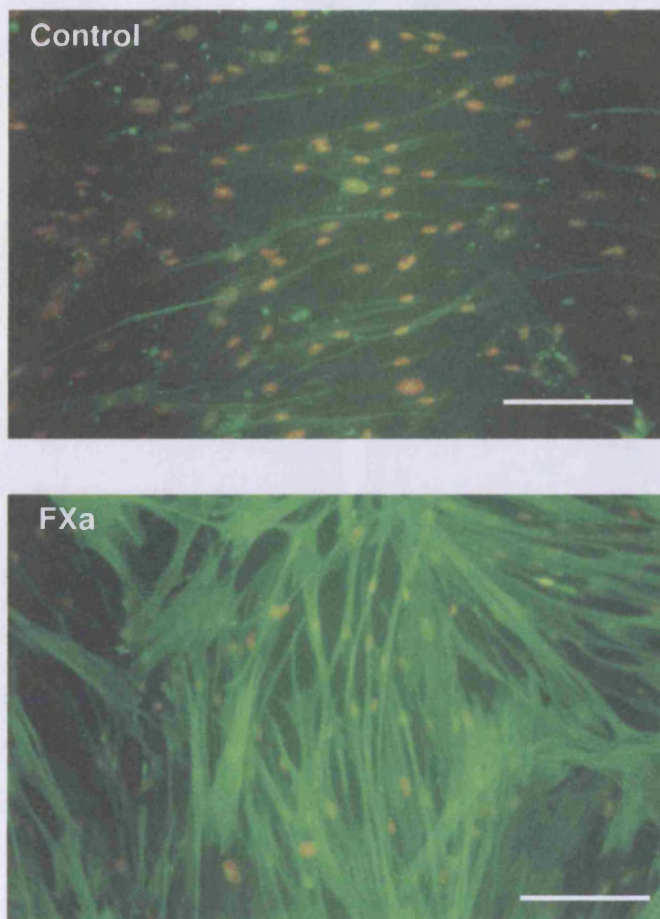
These results show that FXa is able to induce the assembly of stress fibres in human adult as well as foetal lung fibroblasts, which are characteristic of the myofibroblast morphology. Stress fibre assembly is generally thought to directly correlate with the contractile ability of granulation myofibroblasts (Hinz et al, 2001). These data therefore support the notion that FXa mediates the induction of contractile myofibroblast.





**Figure 3.19. Effect of FXa on  $\alpha$ -SMA fibre assembly in pHALFs at 36 hours**

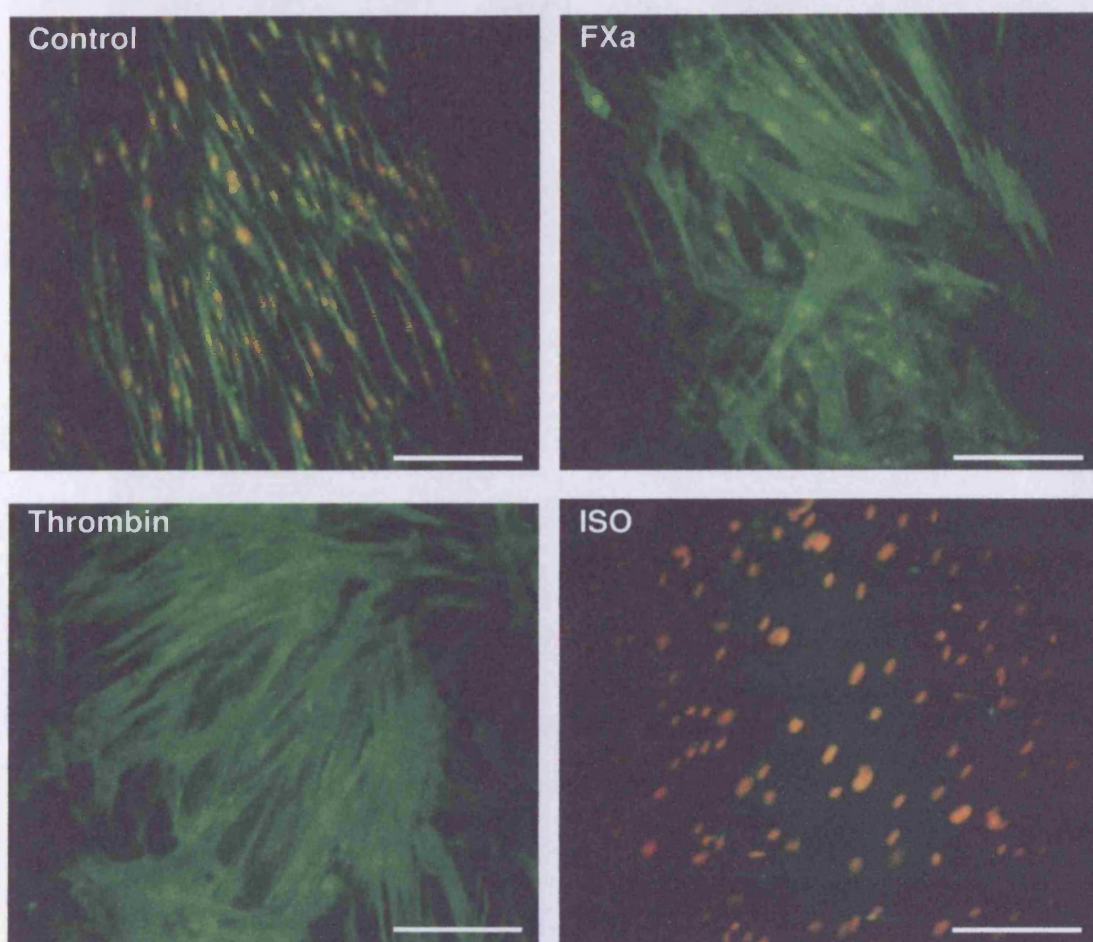
FXa induces the assembly of  $\alpha$ -SMA positive stress fibres in pHALFs characteristic of the myofibroblast phenotype. Cells were grown on chamber slides and exposed to DMEM or FXa for 36 hours. Cells were incubated with anti- $\alpha$ -SMA antibody and fibres were visualized by immunocytofluorescent microscopy.  $\alpha$ -SMA staining is shown in green, nuclei staining with propidium-iodide in red. Isotype control (ISO) showed no staining. Representative images of three separate experiments performed. Scale bar 40  $\mu$ m



**Figure 3.20. Effect of FXa on  $\alpha$ -SMA fibre formation in pHALFs at 96 hours**

FXa induces the assembly of  $\alpha$ -SMA positive stress fibres characteristic of the myofibroblast morphology which persists up to 96hours. Cells were grown on chamber slides and exposed to DMEM or FXa for 96hours. Cells were incubated with anti- $\alpha$ -SMA antibody and fibres were visualized by immunocytofluorescent microscopy.  $\alpha$ -SMA staining is shown in green, nuclei staining with propidium-iodide in red. Isotype control as shown in **Figure 3.19**. Representative images of three separate experiments performed. Scale bars 40 $\mu$ m





**Figure 3.21. Effect of FXa and thrombin on  $\alpha$ -SMA fibre formation in HFL-1 at 36 hours**

FXa induces the assembly of  $\alpha$ -SMA positive stress fibres characteristic of the myofibroblast morphology and very reminiscent of stress fibres induced by thrombin stimulation. Cells were grown on chamber slides and exposed to DMEM or FXa for 36 hours. Cells were incubated with anti- $\alpha$ -SMA antibody and fibres were visualized by immunocytofluorescent microscopy.  $\alpha$ -SMA staining is shown in green, nuclei staining with propidium-iodide in red. Isotype control (ISO) showed no staining. Representative images of three separate experiments performed. Scale bars 40  $\mu$ m.

---

**3.2.8. Summary**

The results described in this section examining the effect of FXa on myofibroblast differentiation showed:

- FXa induces  $\alpha$ -SMA protein levels in a concentration-and time-dependant manner in primary human lung fibroblasts.
- FXa induces  $\alpha$ -SMA protein levels in fetal human and mouse lung fibroblast cell lines.
- FXa regulates  $\alpha$ -SMA expression in primary human lung fibroblasts at the transcriptional level.
- FXa induces the assembly of  $\alpha$ -SMA-positive stress fibres characteristic of the myofibroblast morphology.

In summary, the results in this thesis show that FXa is a potent inducer of myofibroblast differentiation programme in human and mouse lung fibroblasts.

### 3.3 The role of PAR<sub>1</sub> in FXa-induced myofibroblast differentiation

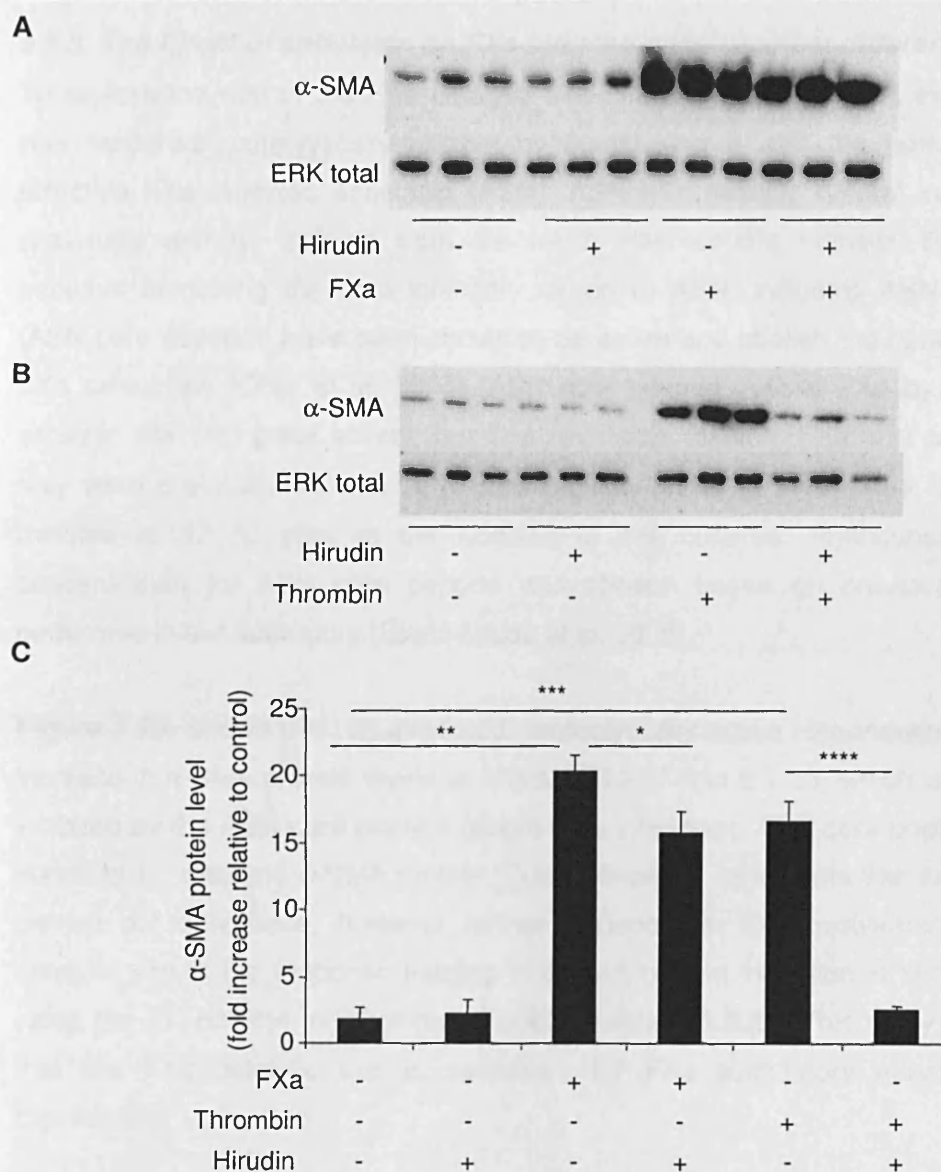
#### 3.3.1. Introduction

Coagulation proteinases such as FXa are known to exert their cellular effects via the proteolytic activation of PARs and FXa can signal via PAR<sub>1</sub> or PAR<sub>2</sub>, or both receptors, depending on cell type and co-factor expression (Blanc-Brude et al, 2005; Ruf et al, 2003). The broad ranges of cellular effects induced by PAR signalling are enabled by the ability of PARs to couple to several different heterotrimeric G proteins, including G $\alpha_q$ , G $\alpha_i$  and G $\alpha_{12/13}$ . To begin to understand the mechanism of action of FXa in the stimulation of myofibroblast differentiation, the following experiments assessed the role of thrombin and FXa catalytic site in  $\alpha$ -SMA expression and explored the contribution of PAR<sub>1</sub> and PAR<sub>2</sub> signalling and differential G protein coupling in this system.

#### 3.3.2. FXa-induced myofibroblast differentiation is independent of thrombin activity

To rule out the possibility that the effect of FXa on  $\alpha$ -SMA expression was mediated via the local generation of thrombin, or contamination of the FXa preparation, FXa-induced  $\alpha$ -SMA induction was investigated in the presence of the highly selective thrombin inhibitor, hirudin. FXa (10nM) and thrombin (positive control; 10nM) were preincubated with hirudin in molar excess (200nM) in serum-free DMEM for 60 minutes at 37 °C prior to the addition to cell cultures. Preincubation time and concentration of hirudin were chosen according to previous work in our laboratory (Blanc-Brude et al, 2005). Control DMEM without inhibitor was incubated under the same conditions.

**Figure 3.22.** shows a representative experiment for the effect of hirudin on fibroblast  $\alpha$ -SMA protein levels in response to FXa and thrombin stimulation. Both FXa and thrombin significantly stimulated  $\alpha$ -SMA protein levels, as assessed at 36hours, by  $20.41 \pm 1.09$  fold and  $16.55 \pm 1.5$  fold respectively. Hirudin had a small, albeit significant, effect on FXa-induced  $\alpha$ -SMA induction (approximately 20% inhibition,  $16.55 \pm 1.5$ ), but it inhibited over 95% of  $\alpha$ -SMA expression stimulated by thrombin ( $2.52 \pm 0.33$ ). This suggests that thrombin exerts only a minor effect on FXa-induced  $\alpha$ -SMA expression.



**Figure 3.22. The effect of hirudin on FXa-induced  $\alpha$ -SMA expression in pHALFs**

Hirudin (200nM) inhibited over 95% of  $\alpha$ -SMA protein levels in response to thrombin (10nM) but only exerted a small (albeit significant) effect on FXa induced  $\alpha$ -SMA protein levels. Hirudin did not affect baseline  $\alpha$ -SMA protein levels.  $\alpha$ -SMA protein was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. **(A)** The effect of hirudin on FXa-induced  $\alpha$ -SMA protein levels. **(B)** The effect of hirudin on thrombin-induced  $\alpha$ -SMA protein levels. **(C)** The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. The same control samples were used within the same experiment to allow for normalisation between blots. Data presented as mean fold increase  $\pm$  s.d and representative of three separate experiments performed with three replicates per group.\* and \*\* and \*\*\*\*,\*\*\*\*\*p<0.001.

### **3.3.3. The Effect of antistasin on FXa-induced myofibroblast differentiation**

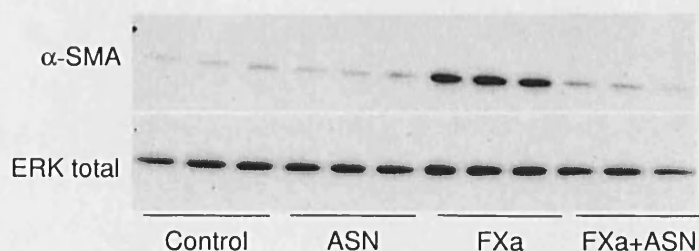
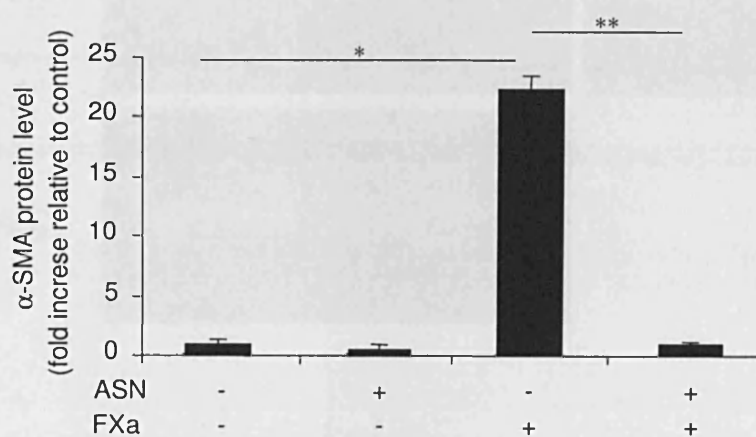
To explore the role of the FXa catalytic site in the induction  $\alpha$ -SMA expression, FXa was rendered proteolytically-inactive by complexing it with the potent and highly selective FXa inhibitor, antistasin (ASN). ASN is a specific natural inhibitor of FXa enzymatic activity, isolated from the leech *Haementeria offinalis*. Short synthetic peptides mimicking the core inhibitory region of ASN, including ASN D-Arg<sup>32</sup>-Pro<sup>38</sup> (ASN core peptide), have been shown to be active and abolish the catalytic activity of FXa selectively (Ohta et al, 1994). ASN core peptide inhibits FXa by binding to its' catalytic site with great affinity, but in a reversible fashion. FXa and control medium only were preincubated with ASN core peptide (1 $\mu$ M) in serum-free DMEM for 120 minutes at 37 °C prior to the addition to cell cultures. Preincubation time and concentration for ASN core peptide was chosen based on previous experiments performed in our laboratory (Blanc-Brude et al, 2005).

**Figure 3.23.** shows that, as expected, proteolytically active FXa induced a significant increase in  $\alpha$ -SMA protein levels at 36hours (22.35 fold  $\pm$  1.15) which was completely inhibited by the ASN core peptide (about 96% inhibition). ASN core peptide alone had no effect on baseline  $\alpha$ -SMA protein. Due to financial constraints this experiment was carried out only twice, however further evidence for the involvement of the FXa catalytic site in the response leading to  $\alpha$ -SMA protein induction is shown in studies using the ZK 807834 inhibitor (see Results **Section 3.5.2.**). This study demonstrates that the FXa catalytic site is necessary for FXa stimulatory effects on  $\alpha$ -SMA expression.

### **3.3.4. Confirmation of PAR<sub>1</sub> and PAR<sub>2</sub> expression at the mRNA level in lung fibroblasts**

FXa is known to signal via PAR<sub>1</sub> or PAR<sub>2</sub> (see Introduction **Section 1.9.**). To begin to examine the potential PAR receptor subtypes involved in mediating FXa-induced stimulatory effects on  $\alpha$ -SMA expression, the relative expression of both receptors was assessed in pHALFs. PAR<sub>1</sub> and PAR<sub>2</sub> expression was analysed in whole cell lysates by RT-PCR; RNA isolated from HFL-1 and MLFs cell lines was analysed in parallel.

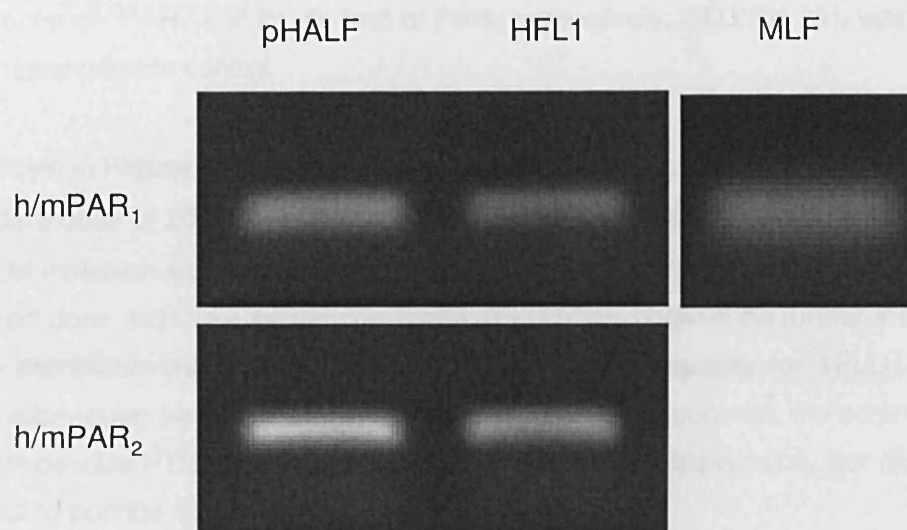
**Figure 3.24.** demonstrates that both receptor subtypes were expressed by pHALFs. As expected, HFL-1 also expressed both subtypes whilst mouse lung fibroblasts only showed a positive signal for PAR<sub>1</sub>.

**A****B**

**Figure 3.23. The effect of antistasin core peptide on FXa-induced  $\alpha$ -SMA protein induction.**

FXa and control medium were preincubated with ASN core peptide (1 $\mu$ M) in serum-free DMEM for 120 minutes at 37 °C prior to addition to cell cultures. **(A)** The effect of ASN core peptide on FXa-induced  $\alpha$ -SMA protein levels.  $\alpha$ -SMA protein was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. ASN core peptide did not affect baseline  $\alpha$ -SMA protein. **(B)** The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Data presented as mean fold increase  $\pm$  s.d and representative of three separate experiments performed with three replicates per group. \*\* $p < 0.001$





**Figure 3.24. Expression of *PAR<sub>1</sub>* and *PAR<sub>2</sub>* in pHALFs, HFL-1 and MLFs**

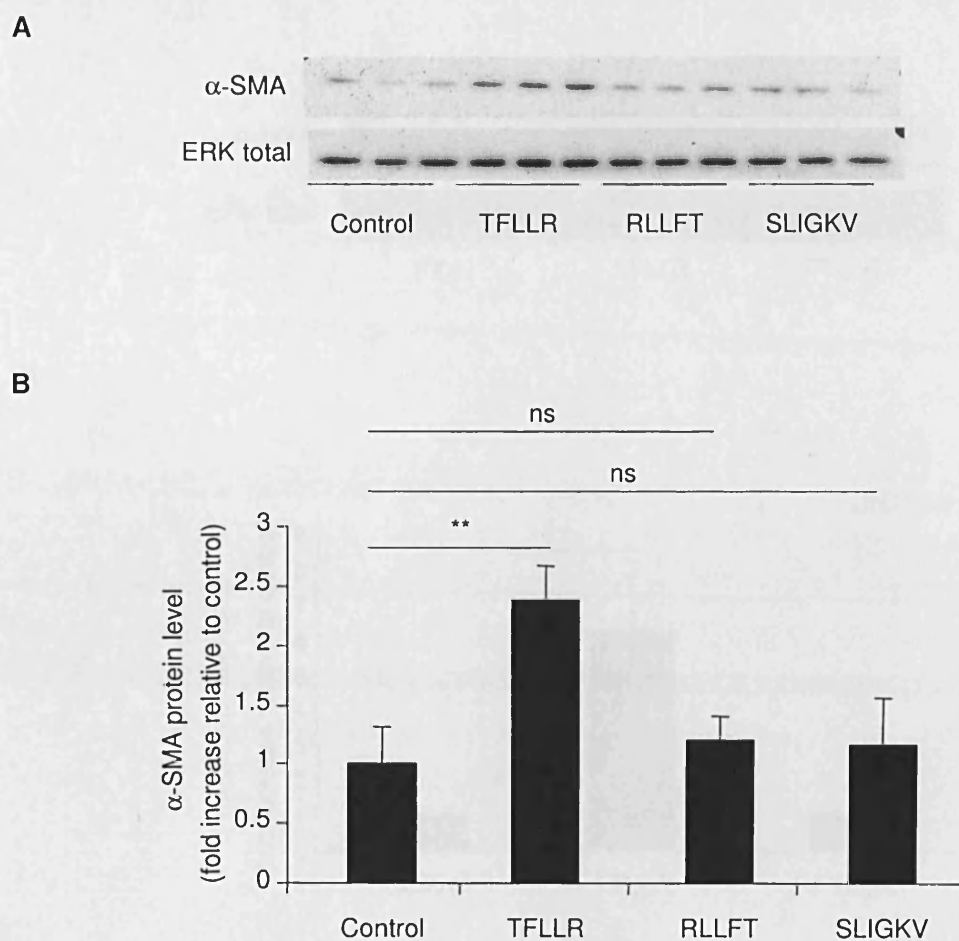
Figure shows the expression of *PAR<sub>1</sub>* and *PAR<sub>2</sub>* as analysed by RT-PCR in whole cell lysates. pHALFs and HFL-1 show expression for both *PAR<sub>1</sub>* and *PAR<sub>2</sub>* whereas MLFs only express the *PAR<sub>1</sub>* receptor subtype. This image is representative of three replicates performed in this experiment.

### **3.3.5. PAR<sub>1</sub> but not PAR<sub>2</sub> activation is sufficient for fibroblast to myofibroblast differentiation.**

Having demonstrated that pHALFs express both PAR<sub>1</sub> and PAR<sub>2</sub> at the mRNA level, the next set of experiments examined whether activation of PAR<sub>1</sub> or PAR<sub>2</sub> or both was necessary and/or sufficient for the induction of  $\alpha$ -SMA expression in pHALFs. Selective PAR<sub>1</sub> and PAR<sub>2</sub> activation was achieved by stimulating pHALFs with synthetic activating peptides TFLLRn-NH<sub>2</sub> and SLIGKV-NH<sub>2</sub> corresponding to the tethered ligand sequence of PAR<sub>1</sub> and its it's and of PAR<sub>2</sub> respectively. FTLLRN-NH<sub>2</sub> was used as a scrambled peptide control.

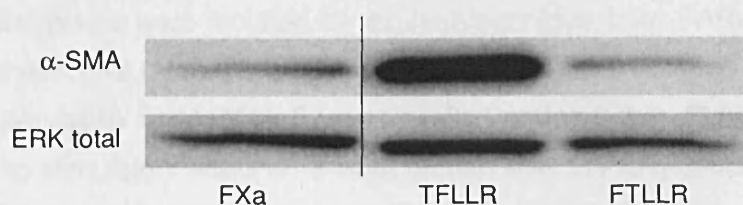
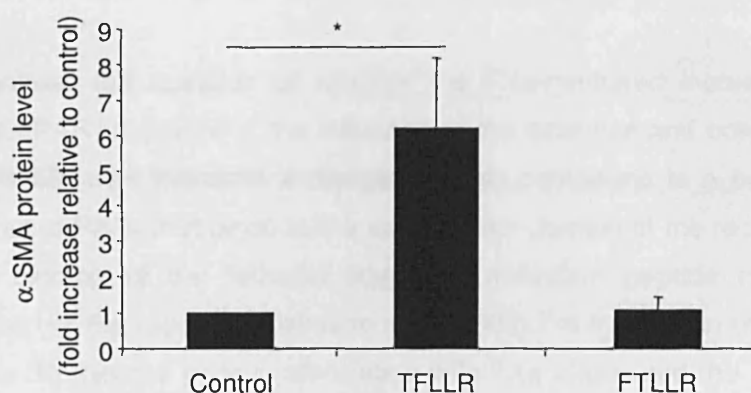
As shown in **Figure 3.25.**, TFLLR-NH<sub>2</sub> significantly increased  $\alpha$ -SMA protein levels at a standard dose of 200 $\mu$ M (2.38 fold  $\pm$  0.29) but was less efficient when compared to the level of induction seen following stimulation with FXa in previous experiments. Further detailed dose response experiments with TFLLR-NH<sub>2</sub> showed no further increase in  $\alpha$ -SMA expression suggesting that maximal stimulatory capacity for TFLLR-NH<sub>2</sub> on  $\alpha$ -SMA expression had been achieved (data not shown). In contrast, the scrambled PAR<sub>1</sub> control peptide FTLLR-NH<sub>2</sub> exerted no effect on  $\alpha$ -SMA expression, nor did the PAR<sub>2</sub> activating peptide SLIGKV-NH<sub>2</sub>.

Induction of  $\alpha$ -SMA expression by PAR<sub>1</sub> activating peptides was also confirmed in MLFs; SLIGKV-NH<sub>2</sub> was not used since these cells lack PAR<sub>2</sub> expression (**Figure 3.26.**). TFLLR-NH<sub>2</sub> induced a 5.33 fold  $\pm$  2.16 increase in  $\alpha$ -SMA protein levels (compared to FXa which stimulated  $\alpha$ -SMA by 6.19 fold  $\pm$  1.98); FTLLR-NH<sub>2</sub> had no effect. These data show that PAR<sub>1</sub>, but not PAR<sub>2</sub> activation, is sufficient for  $\alpha$ -SMA induction.



**Figure 3.25. The effect of TFLLR-NH<sub>2</sub>, SLIGKV-NH<sub>2</sub> and FTLLR-NH<sub>2</sub> on FXa-induced α-SMA protein induction in pHALFs.**

**(A)** The synthetic PAR<sub>1</sub> (TFLLR-NH<sub>2</sub>, 200μM)-and PAR<sub>2</sub> (SLIGKV-NH<sub>2</sub>, 200μM) activating peptides reveal that PAR<sub>1</sub> activation is sufficient for the induction of α-SMA and that PAR<sub>2</sub> activation does not induce α-SMA expression. α-SMA protein was detected using an anti-α-SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. **(B)** The relative increase in α-SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Data representative of three separate experiments performed with three replicates in each group. \*\*p=0.0002

**A****B**

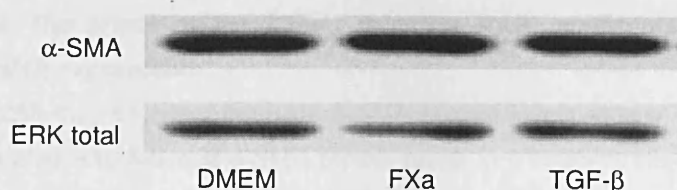
**Figure 3.26. The effect of TFLLR-NH<sub>2</sub> and FTLLR-NH<sub>2</sub> on FXa-induced  $\alpha$ -SMA protein induction in MLFs**

(A) The effect of synthetic peptide agonists for PAR<sub>1</sub> (TFLLR-NH<sub>2</sub>, 200 $\mu$ M) and its scrambled peptide control FTLLR-NH<sub>2</sub> (200 $\mu$ M) on  $\alpha$ -SMA protein levels in MLFs.  $\alpha$ -SMA protein was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. Line indicates that protein bands were not positioned directly adjacent to each other on the blot. (B) The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Data representative of three separate experiments performed with three replicates in each group. \* $p < 0.001$

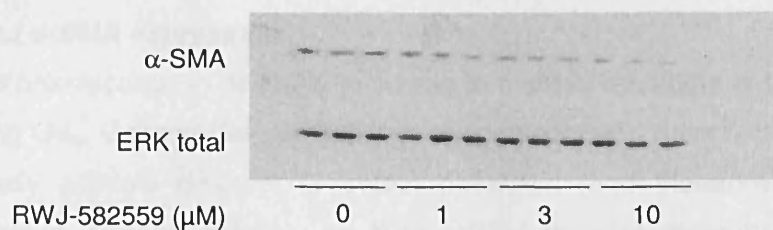
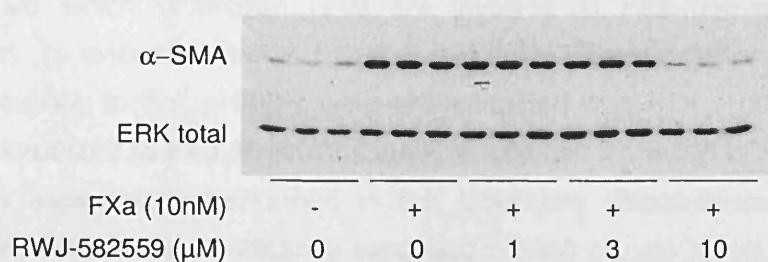
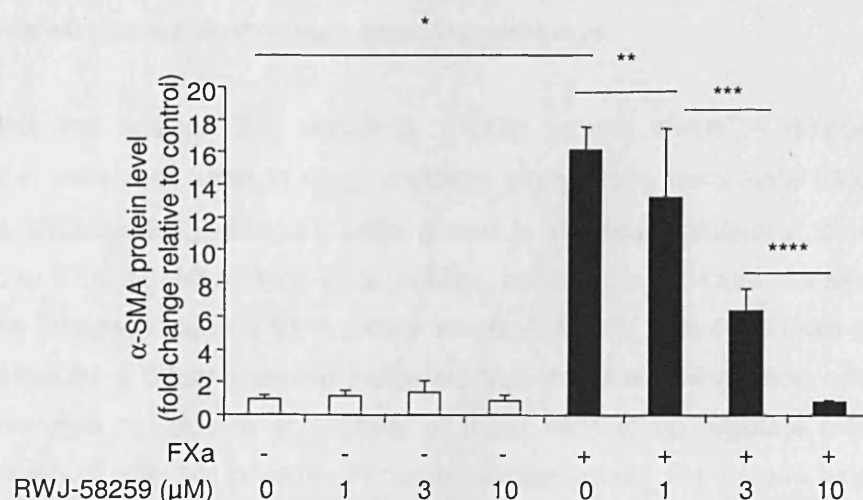
### 3.3.6. PAR<sub>1</sub> activation is necessary for FXa-induced myofibroblast differentiation

To examine whether PAR<sub>1</sub> was necessary for FXa-induced  $\alpha$ -SMA expression, primary mouse lung fibroblasts were isolated by explant technique from PAR<sub>1</sub>-deficient-mice and stimulated with FXa (10nM) and the positive control TGF- $\beta$  (2ng/ml) under identical conditions as previously used for MLF (see Results **Section 3.2.4**). **Figure 3.27.** shows that FXa had no stimulatory effect on  $\alpha$ -SMA protein level in PAR<sub>1</sub>-deficient fibroblasts. However TGF- $\beta$  stimulation also failed to induce an increase. It was further noted that baseline expression of  $\alpha$ -SMA was markedly higher compared to that observed for fibroblasts in previous experiments, suggesting that PAR<sub>1</sub>-deficient fibroblasts were already expressing a maximum level of  $\alpha$ -SMA and could therefore not be used for the purpose of this experiment.

In order to answer the question of whether the FXa-mediated increase in  $\alpha$ -SMA expression was PAR<sub>1</sub> dependent, the influence of the selective and potent inhibitor of PAR<sub>1</sub>, RWJ-58259, was therefore investigated. This compound is a highly selective small antagonist of PAR<sub>1</sub> that binds to the extracellular domain of the receptor, thereby inhibiting the binding of the tethered ligand or activation peptide to the second extracellular loop of the receptor (Damiano et al, 2003). Pre-incubation of pHALFs with RWJ-58259 for 30 minutes prior to stimulation with FXa attenuated the FXa-mediated increase in  $\alpha$ -SMA protein levels in a concentration-dependent manner from 3 $\mu$ M onwards (about 61% inhibition; **Figure 3.28.**) At the highest concentration of RWJ-58259 (10 $\mu$ M) the increase in  $\alpha$ -SMA protein levels was blocked by about 94%. This compound had no effect on basal  $\alpha$ -SMA protein. These data confirm that FXa exerts its stimulatory effect on  $\alpha$ -SMA expression via a PAR<sub>1</sub>-dependent mechanism.



**Figure 3.27. Effect of FXa and TGF- $\beta$  on PAR<sub>1</sub>-deficient mouse lung fibroblasts.** Lung fibroblasts were isolated from PAR<sub>1</sub> deficient mice prior to stimulation with FXa (10nM) or TGF- $\beta$  (2ng/ml). FXa and TGF- $\beta$  had no effect on  $\alpha$ -SMA protein levels.  $\alpha$ -SMA protein was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. Data representative of three separate experiments performed.

**A****B****C**

**Figure 3.28. The effect of the highly selective  $PAR_1$  antagonist, RWJ-58259 on FXa-induced  $\alpha$ -SMA expression.**

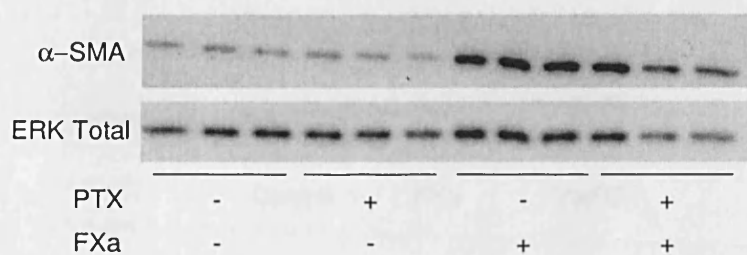
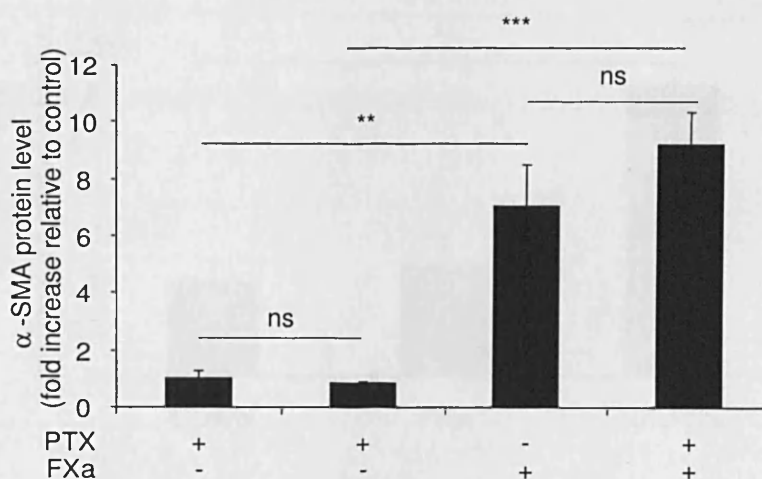
(A) RWJ-58259 did not affect baseline  $\alpha$ -SMA protein levels at any concentration. (B) RWJ-58259 attenuated FXa-induced  $\alpha$ -SMA protein levels in a concentration-dependent manner.  $\alpha$ -SMA was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. (C) The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry.  $\alpha$ -SMA protein levels were detected using an anti- $\alpha$ -SMA antibody and blots were reprobed with total ERK2 for protein loading control. The same control samples were used within the same experiment to allow for normalisation between blots. All data is representative of three separate experiments with three replicates in each experimental group expressed as mean fold increase  $\pm$  s.d, \*and \*\*\*and \*\*\*\* $p < 0.001$ , \*\* $p = 0.029$ .

### 3.3.7. Effect of pertussis toxin (PTX) and the PAR<sub>1</sub>-Gα<sub>q</sub> inhibitor (Q94) on FXa-Induced α-SMA expression

PAR<sub>1</sub> is promiscuous in its ability to couple to multiple members of the G protein family, including Gα<sub>q</sub>, Gα<sub>i</sub> and Gα<sub>12/13</sub>. Different agonists of PAR<sub>1</sub> have recently been shown to selectively activate different G-protein pathways (and therefore induce differential downstream cellular effects), by their ability to alter receptor/G protein binding (McLaughlin et al, 2005b). The following set of experiments therefore aimed to investigate which G protein units are involved in FXa-mediated α-SMA protein induction. To evaluate whether FXa-induced α-SMA protein levels were dependent on PAR<sub>1</sub> coupling to Gα<sub>i</sub>, pHALFs were pre-incubated with PTX (100 ng/ml) for 3 hours prior to exposure to FXa. Preincubation time and concentration of PTX were based on previous experiments performed in our laboratory (Blanc-Brude et al, 2005). As expected, FXa alone significantly increased α-SMA protein levels (6.99 fold ± 1.48). PTX exerted no inhibitory effect on the induction of α-SMA protein levels (**Figure 3.29.**) suggesting that PAR<sub>1</sub>-mediated α-SMA expression is independent of PAR<sub>1</sub> coupling to Gα<sub>i</sub> and its associated downstream signalling pathways.

To assess the role of Gα<sub>q</sub> signalling, α-SMA protein levels in response to FXa stimulation were measured in Gα<sub>q11</sub>-deficient mouse lung fibroblasts (Gα<sub>q11</sub> -/-) (see Methods **Section 2.4.**). Gα<sub>q11</sub>-/- were grown in identical conditions to pHALFs and exposed to FXa (10nM) or 10% FCS (positive control) for 36 hours. As seen in **Figure 3.30.** FXa failed to induce α-SMA protein levels, however 10% FCS used as a positive control induced a 3-fold increase indicating that the lack of induction seen with FXa stimulation was not due to an inability of these cells to up regulate α-SMA *per se*. Unfortunately, it was not possible to obtain corresponding WT mouse lung fibroblasts from litter mates to exclude the possibility that fibroblasts isolated from this mouse strain are unable to respond to FXa in general. A pharmacological approach was therefore used to evaluate the potential involvement of Gα<sub>q</sub>. This question was addressed using the novel inhibitor Q94 which selectively blocks the PAR<sub>1</sub> interaction with Gα<sub>q</sub> (Caden Biosciences). Pre-incubation of pHALFs with Q94 for 3 hours prior to stimulation with FXa (10nM) blocked the induction of α-SMA protein levels in a concentration-dependent manner from 0.1μM onwards (about 20% inhibition, **Figure 3.31**). This compound had no effect on basal α-SMA protein. These data suggest that FXa-mediated α-SMA expression via PAR<sub>1</sub> is independent of receptor coupling to Gα<sub>i</sub> and dependent on receptor coupling to Gα<sub>q</sub>.

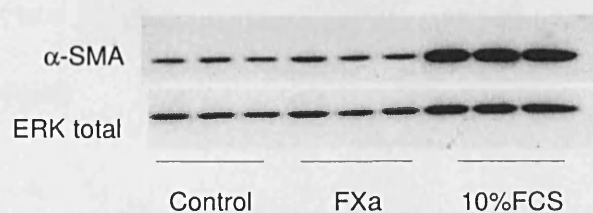
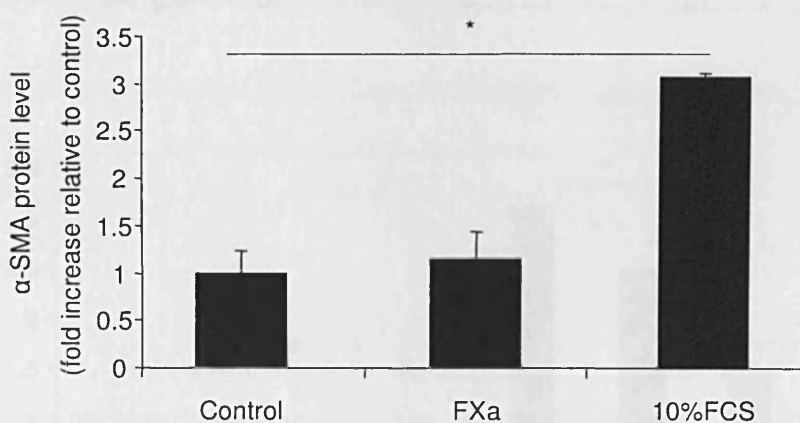


**A****B**

**Figure 3.29. Effect of PTX on FXa-induced α-SMA expression.**

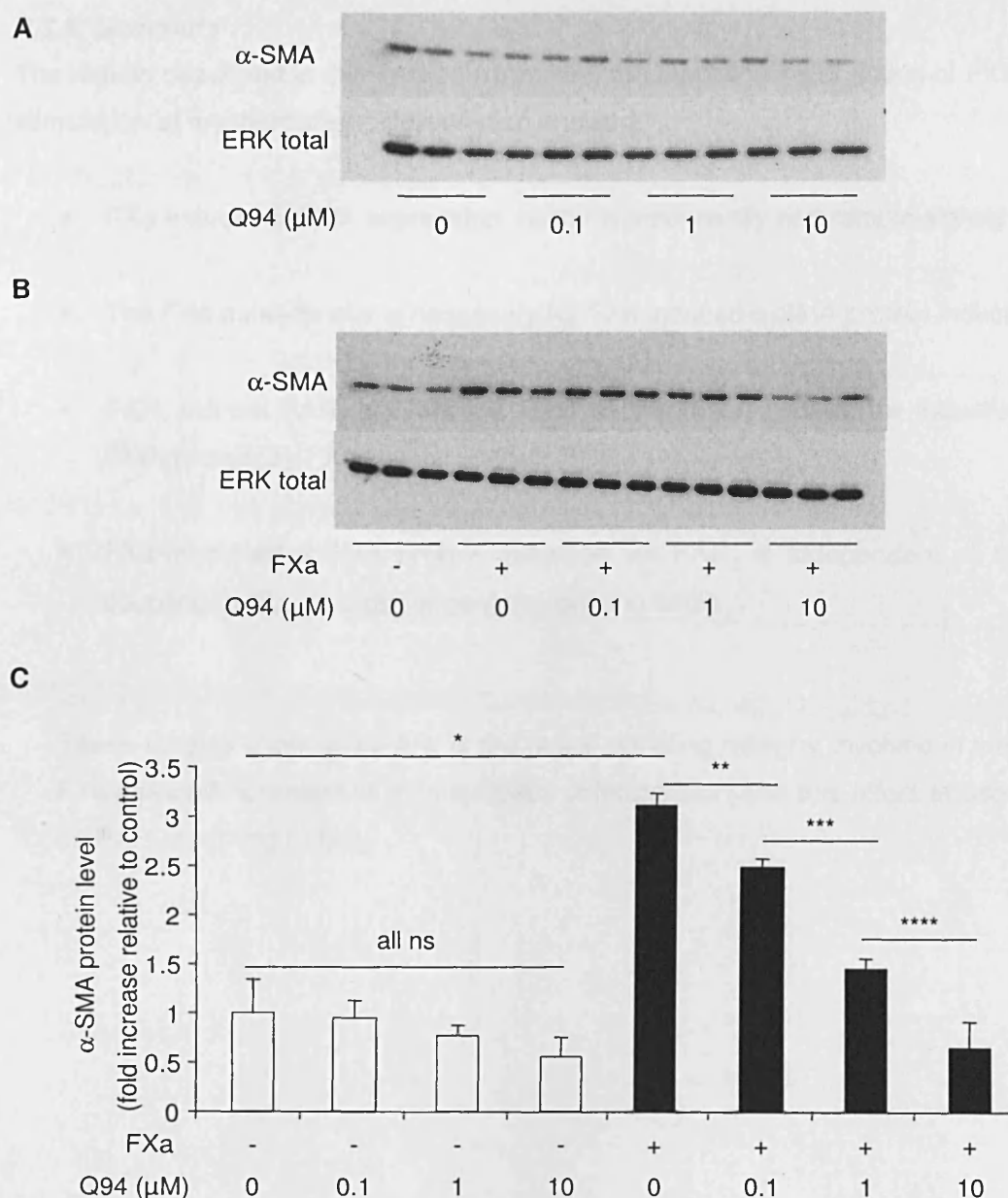
Incubation of pHALFs with PTX (100ng/ml) blocked FXa-induced α-SMA protein levels in a concentration dependent manner. **(A)** α-SMA protein levels were detected using an anti-α-SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. PTX did not affect baseline α-SMA protein **(B)** The relative increase in α-SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. All data is representative of three separate experiments with three replicates in each experimental group expressed as mean fold increase  $\pm$  s.d, \* $p$  = 0.0023, \*\* $p$  < 0.001



**A****B**

**Figure 3.30. Effect of FXa stimulation on α-SMA protein induction in  $G_{\alpha q/11}$ -deficient mouse lung fibroblasts.**

(A) α-SMA protein levels were detected using an anti-α-SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. PTX did not affect baseline α-SMA protein (B) The relative increase in α-SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. All data expressed as mean fold increase  $\pm$  s.d, \* $p < 0.001$



**Figure 3.31. Effect of the selective  $G\alpha_q$  antagonist Q94 on FXa-induced  $\alpha$ -SMA induction**

(A) Q94 did not affect baseline  $\alpha$ -SMA protein levels at any concentration. (B) Q94 attenuated FXa-induced  $\alpha$ -SMA protein levels in a concentration-dependent manner.  $\alpha$ -SMA was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. (C) The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Q94 blocked up to 95% of FXa induced  $\alpha$ -SMA protein levels at its highest concentration. The same control samples were used within the same experiment to allow for normalisation between blots. All data is representative of three separate experiments with three replicates in each experimental group expressed. \*and\*\*and\*\*\*and\*\*\*\* $p < 0.001$ .

---

**3.3.8. Summary**

The results described in this section examining the mechanisms of action of FXa in the stimulation of myofibroblast differentiation showed:

- FXa induces  $\alpha$ -SMA expression mostly independently of thrombin activity.
- The FXa catalytic site is necessary for FXa-induced  $\alpha$ -SMA protein induction.
- PAR<sub>1</sub> but not PAR<sub>2</sub> activation is necessary and sufficient for the induction of  $\alpha$ -SMA protein by FXa.
- FXa-mediated  $\alpha$ -SMA protein induction via PAR<sub>1</sub> is independent of receptor coupling to G $\alpha_i$ , and dependent on coupling to G $\alpha_q$ .

These studies show that PAR<sub>1</sub> is the major signaling receptor involved in mediating FXa-induced fibroblast to myofibroblast differentiation and this effect is dependent on PAR<sub>1</sub> coupling to G $\alpha_q$ .

### 3.4. The role of TGF- $\beta$ in FXa-induced myofibroblast differentiation

#### 3.4.1. Introduction

Previous time-course experiments revealed that  $\alpha$ -SMA induction at both the mRNA and protein level was a delayed response to FXa raising the possibility that FXa exerts its effects via the autocrine production and/or activation of a secondary mediator. TGF- $\beta$  is one of the most potent known inducers of the myofibroblast differentiation programme and signals in fibroblasts via the activation of the activin-like kinase 5 (ALK-5) TGF- $\beta$  type 1 receptor, which triggers the SMAD signalling pathway by phosphorylating SMAD-2 and -3. Thrombin has previously been shown to increase expression and/or release of TGF- $\beta$  from vascular smooth muscle cells (Bachhuber et al, 1997), and kidney mesangial and epithelial cells (Yamabe et al, 1997; Tsunoda et al, 2001; Shirato et al, 2003). The following section therefore explored the hypothesis that FXa is mediating its effects on  $\alpha$ -SMA expression via a TGF- $\beta$ -dependent mechanism.

#### 3.4.2. FXa does not regulate TGF- $\beta$ mRNA levels

Following cellular synthesis, TGF- $\beta$  is released as biologically inactive latent-TGF- $\beta$ , which comprises a complex of a latency-associated peptide (LAP) and a 25kDa mature TGF- $\beta$  dimer (see Introduction Section 1.6.2.). There is compelling evidence that TGF- $\beta$  bioavailability is regulated at the production level of the cytokine and/or at the level of activation of its latent form. To examine if FXa influenced TGF- $\beta$  production, the effect of FXa on TGF- $\beta_1$  gene expression in pHALFs was assessed by real-time RT-PCR. pHALFs were grown to sub-confluency in 6-well plates and stimulated with FXa (10nM) over time up to 24 hours (the time point at which the first significant increase in  $\alpha$ -SMA mRNA levels can be observed) in identical conditions to those used for  $\alpha$ -SMA stimulation.

As seen in **Figure 3.32**, FXa caused no apparent change in TGF- $\beta$  mRNA levels at any time point tested, suggesting that FXa does not regulate TGF- $\beta$  production at the transcriptional level.

### 3.4.3. *TFLLR-NH<sub>2</sub> induces activation of TGF- $\beta$*

The next set of experiments determined whether FXa acts via activation of latent TGF- $\beta$  rather than via a transcriptional mechanism. Active TGF- $\beta$  levels were assessed using a bioassay based on a transformed Mink lung epithelial cell (tMLEC) and pHALF co-culture system where fibroblast-derived active TGF- $\beta$  is detected as an increase in luciferase activity by tMLEC which express luciferase under the control of the TGF- $\beta$ -responsive PAI-1 promoter (see Methods **Section 2.21.**).

In these experiments, it was not possible to test the effect of FXa on TGF- $\beta$  activity. FXa induced a significant increase in luciferase activity at 10 and 25 nM concentration ( $1.22 \pm 0.06$ -and  $1.57 \pm 0.11$ -fold, respectively), however the relative fold increase was not inhibited by the pan-specific TGF- $\beta$  blocking antibody 1D11 (100ng,  $1.14 \pm 0.1$ -and  $1.42 \pm 0.17$ -fold, respectively). To exclude the possibility that an antibody blocking strategy is not effective in this system, the effect of a small molecule inhibitor of the TGF- $\beta$  type I receptor (ALK-5), SB431542 (Callahan et al, 2002) was also examined. SB431542 similarly had no effect on FXa-induced increase in luciferase activity ( $1.22 \pm 0.11$ -and  $2.06 \pm 0.15$ -fold respectively), suggesting that FXa is influencing baseline luciferase activity in this assay (see **Figure 3.33.**). The experiments were therefore performed with the selective PAR<sub>1</sub> agonist, TFLLR-NH<sub>2</sub> and its scrambled peptide control FTLLR-NH<sub>2</sub>.

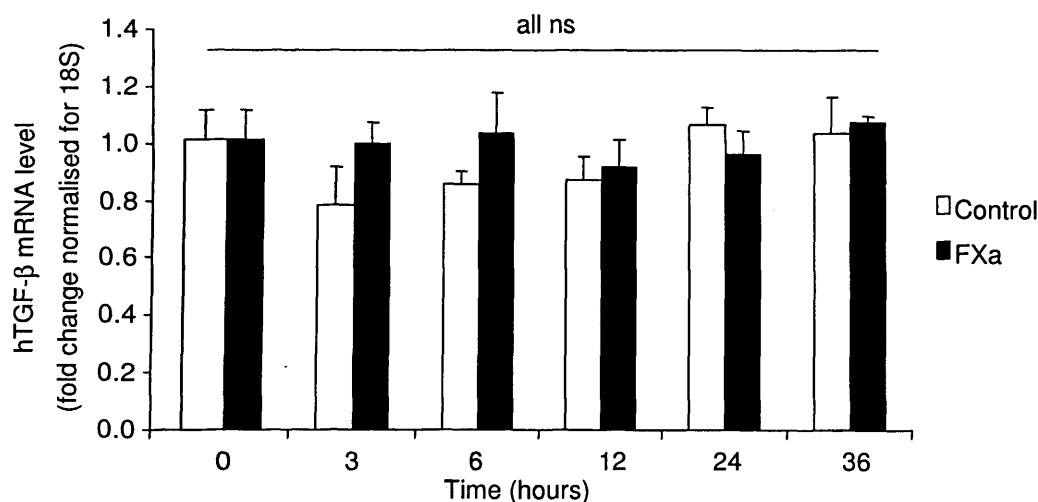
Luciferase activity was significantly increased in response to exogenous TFLLR-NH<sub>2</sub> used at a standard dose of 200 $\mu$ M compared with media control-treated cell cultures ( $1169 \pm 68.67$  versus  $857 \pm 98.6$ -fold increase, respectively **Figure 3.34.**). Moreover, this increase in luciferase activity was completely inhibited by the pan-specific TGF- $\beta$  blocking antibody 1D11 (100 $\mu$ g/ml;  $774.25 \pm 107.72$ -fold increase), indicating that the increase in tMLEC luciferase activity was due to the presence of active TGF- $\beta$ . As expected, FTLLR-NH<sub>2</sub> had no stimulatory effect. Furthermore increased TGF- $\beta$  activity was only observed when pHALFs were grown in co-culture with tMLEC rather than in a single culture system where tMLEC are stimulated with conditioned media taken from pHALFs (data not shown), that close cell contact between the two cell types (the effector and the reporter cell line) is essential.

### 3.4.4. *FXa induces SMAD2/3 phosphorylation in pHALFs*

To determine the time course of PAR<sub>1</sub>-mediated TGF- $\beta$  activation, FXa stimulation of TGF- $\beta$ -induced signalling pathways was assessed as a measure of TGF- $\beta$  activity. Active TGF- $\beta$  causes activation of ALK5 leading to phosphorylation of SMAD2/3 (see

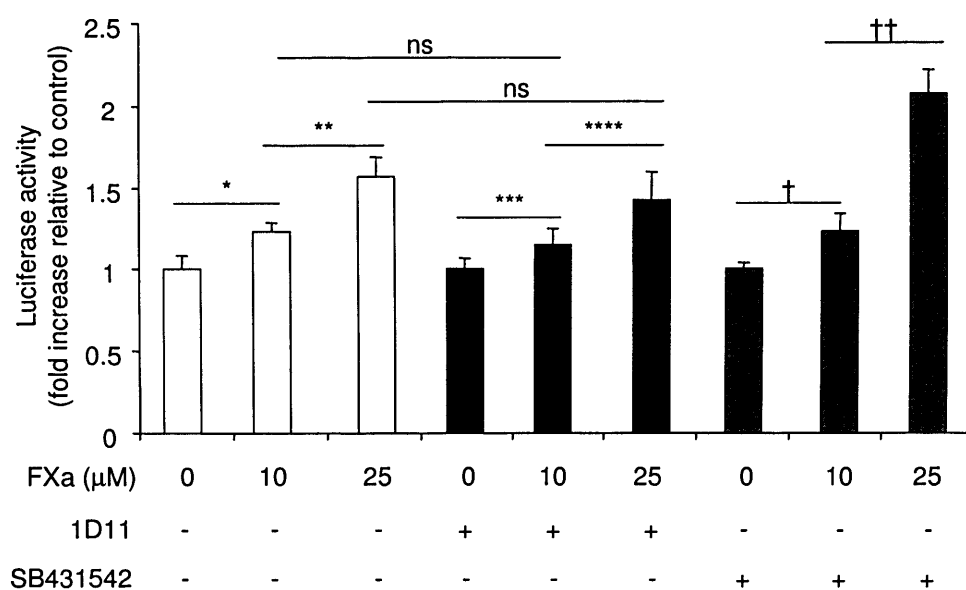
Introduction **Section 1.6.1.**). The effect of FXa on SMAD2/3 phosphorylation in pHALFs was therefore examined. Cells were exposed to a single dose of FXa (10nM) in serum-free conditions and SMAD2/3 phosphorylation was determined from 1 hour up to 24 hours by western blotting.

As shown in **Figure 3.35. (A)**, the anti-phospho SMAD2/3 antibody recognized a faint 58-kDa band in unstimulated pHALFs. The intensity of this band was highly increased in cells exposed to FXa. Densitometric analysis revealed that FXa stimulated phosphorylation of SMAD2/3 from 6 hours onwards (5.9-fold at 6 hours; **Figure 3.35. (B)**). Control fibroblasts treated with DMEM only, showed no significant increase in  $\alpha$ -SMA over time. The response was maximal at 12 hours with an observed increase of 12.21-fold over medium control. Similar kinetics in phsphoSMAD2/3 protein levels were observed in six separate experiments that were performed with one replicate per experimental group. These experiments reveal that FXa increased SMAD2 phosphorylation with the earliest detectable signal observed 6 hours following FXa stimulation



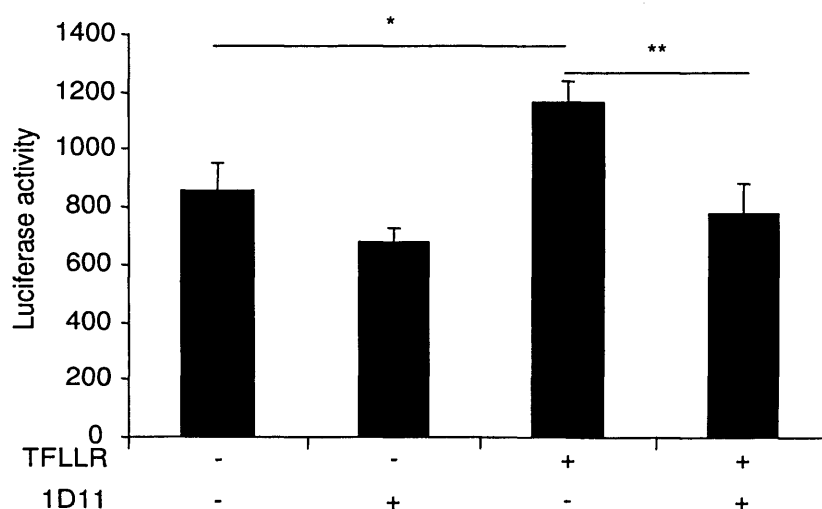
**Figure 3.32. FXa does not stimulate TGF- $\beta$  gene expression.**

Figure shows the effect of FXa (10nM) stimulation on TGF- $\beta$  mRNA levels in pHALFs over time, up to 36 hours. TGF- $\beta$  mRNA levels were determined by real-time RT-PCR. Representative figure of two separate experiments performed with three replicates in each experimental group. Data expressed as fold increase over media control  $\pm$  s.d.



**Figure 3.33. FXa-induced luciferase activity is not blockable by 1D11 and SB431542**

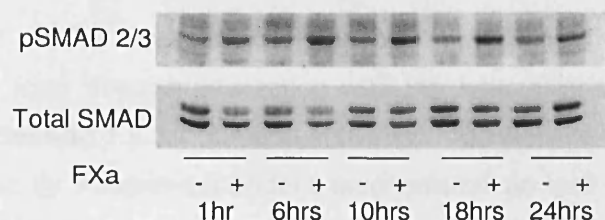
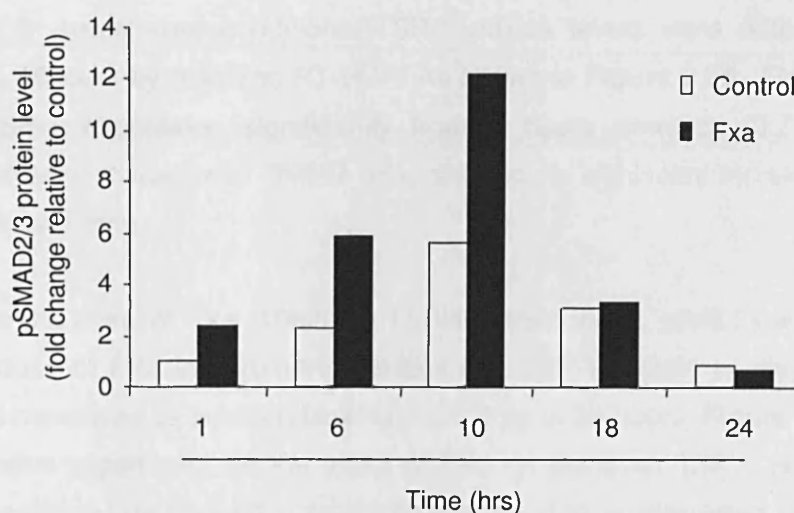
Graph showing the effect of FXa on luciferase activity measured using a tMLEC-fibroblast co-culture system (1:1) where fibroblast-derived active TGF- $\beta$  is detected as an increase in luciferase activity by tMLEC expressing luciferase under the control of the TGF- $\beta$ -responsive PAI-1 promoter. FXa (10 and 25 nM) induced a significant increase in luciferase activity that was not blockable by the TGF- $\beta$  blocking antibody 1D11 (100 $\mu$ g/ml) nor by the ALK5 inhibitor SB431542 (10 $\mu$ M). Data expressed as fold increase over media control  $\pm$  s.d. \*and \*\*and\*\*\*and\*\*\*\*p<0.001



**Figure 3.34. Effect of TFLLR-NH<sub>2</sub> on TGF- $\beta$  activity.**

Graph showing the effect of TFLLR on luciferase activity measured using a tMLEC-fibroblast co-culture system (1:1) where fibroblast-derived active TGF- $\beta$  is detected as an increase in luciferase activity by tMLEC expressing luciferase under the control of the TGF- $\beta$ -responsive PAI-1 promoter. TFLLR induced a significant increase in luciferase activity which was completely blocked by 1D11 (100 $\mu$ g/ml). Data expressed as fold increase over media control  $\pm$  s.d.\*and \*\*p<0.001



**A****B**

**Figure 3.35. Time course experiment for phosphorylation of SMAD 2/3 by FXa.**

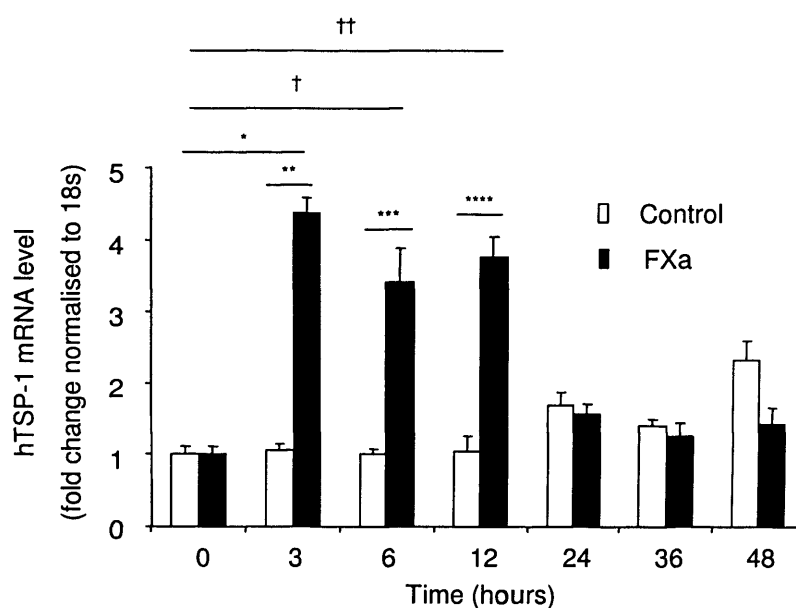
FXa increased SMAD2/3 phosphorylation with the earliest detectable signal observed 6 hours following FXa stimulation. **(A)** Phosphorylated SMAD2/3 was detected using an anti-phospho-SMAD2/3 antibody and blots were then stripped and reprobbed with total SMAD as protein loading control. **(B)** The relative increase in phospho SMAD protein levels normalised to total SMAD protein levels was determined by semi-quantitative densitometry. All data are representative of six separate experiments with one replicate in each experimental group. Data expressed as fold increase over media control  $\pm$  s.d.

### **3.4.5. FXa stimulates thrombospondin-1 (TSP-1) expression at the mRNA and protein level**

The activation of TGF- $\beta$  involves the conversion of the latent precursor to its biologically active form through interaction with the latency associate peptide (LAP) (see Introduction **Section 1.6.3.**). Several activation mechanisms have been described, including activation by integrin-dependent mechanisms as well as interaction of the LAP with TSP-1. PAR<sub>1</sub> activation is a strong inducer of TSP-1 expression in endothelial cells (Riewald and Ruf, 2005). To begin to examine if TSP-1 mediates activation of latent TGF- $\beta$  by FXa, first the effect of FXa stimulation on TSP-1 expression in pHALFs was examined at the mRNA as well as at the protein level.

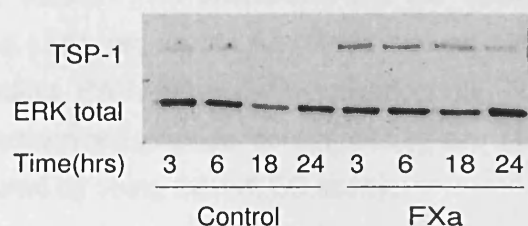
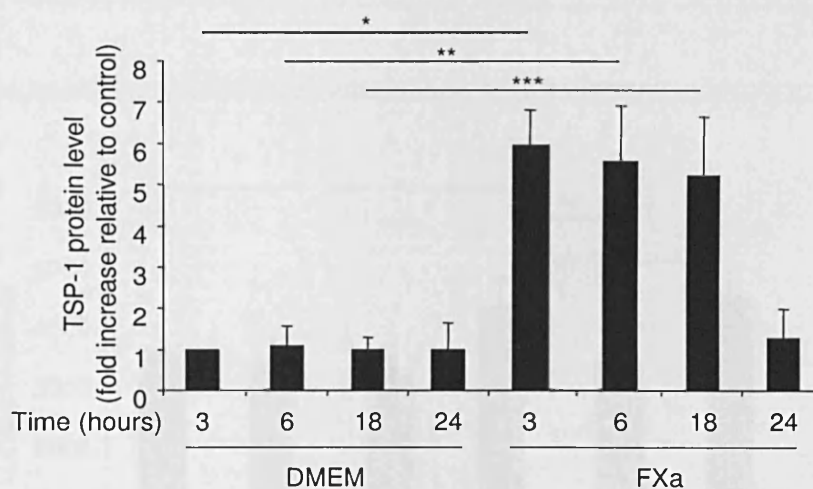
pHALFs were grown to sub-confluency in 6-well plates and exposed to a single dose of FXa (10nM) in serum-free conditions. TSP-1 mRNA levels were determined from 3hours up to 48hours by real time RT-PCR. As shown in **Figure 3.36.**, FXa stimulated fibroblast TSP-1 expression significantly from 3 hours onwards ( $3.74 \pm 1.19$ -fold). Control fibroblasts, treated with DMEM only, showed no significant increase in TSP-1 mRNA levels over time.

To determine the effect of FXa (10nm) on TSP-1 protein levels, pHALFs were exposed to a single dose of FXa in serum-free DMEM and TSP-1 protein in whole cell layer extracts was measured by western blotting, from 3 up to 24 hours. **Figure 3.37.** shows a representative experiment for the effect of FXa on fibroblast TSP-1 protein levels. The TSP-1 antibody recognized a faint 170-kDa band in unstimulated pHALFs. The intensity of this band was highly increased in cells exposed to FXa. Densitometric analysis of the protein bands revealed that FXa stimulated fibroblast TSP-1 protein levels significantly from 3hours onwards ( $5.93 \pm 0.85$ -fold at 3 hours). Control fibroblasts treated with DMEM, showed no significant increase in TSP-1 protein over time. This time course was reminiscent of that observed for TSP-1 mRNA levels following FXa stimulation. These data show that FXa stimulates TSP-1 expression in pHALFs at both the mRNA and at the protein level.



**Figure 3.36. Effect of FXa stimulation on TSP-1 mRNA levels**

Figure shows the effect of FXa (10nM) stimulation on TSP-1 mRNA levels in pHALFs over time, up to 48 hours. TSP-1 mRNA levels were determined by real-time RT-PCR. Representative figure of three separate experiments performed with three replicates in each experimental group. Data expressed as fold increase over media control  $\pm$  s.d relative to time 0 hours. \*and\*\*and\*\*\*and\*\*\*\*and†and††  $p < 0.001$

**A****B**

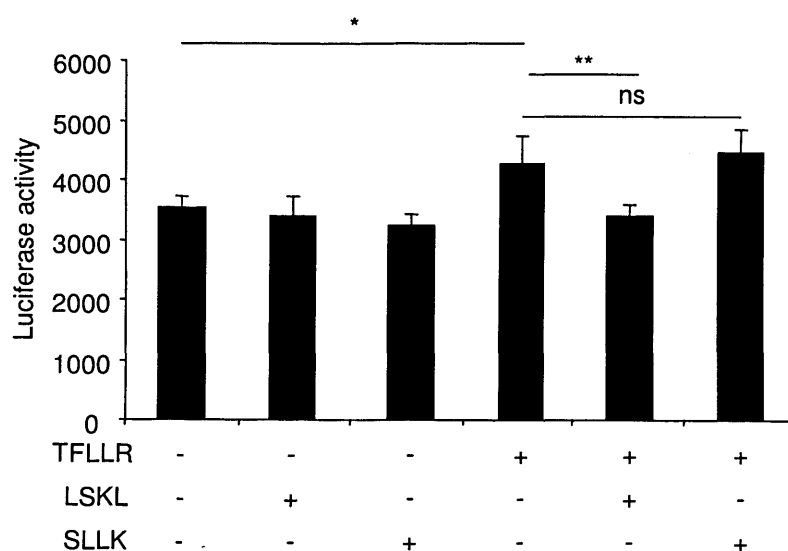
**Figure 3.37. Effect of FXa on TSP-1 protein level in pHALFs**

FXa increased TSP-1 protein levels with the earliest detectable signal observed 3 hours following FXa stimulation. **(A)** TSP-1 protein was detected using an anti-TSP-1 antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. **(B)** The relative increase in TSP-1 protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Representative experiment for three separate experiments performed. All data represented as mean fold increase over media control  $\pm$  s.d relative to control at time 3 hours. \*and \*\*and \*\*\* $p < 0.001$

### 3.4.6. PAR<sub>1</sub> mediates TGF- $\beta$ activation in a TSP-1-dependent manner

TSP-1 activates TGF- $\beta$  by interacting with the LSKL region of LAP (reviewed in (Young and Murphy-Ullrich, 2004)). This interaction can be inhibited by synthetic peptides corresponding to the LSKL region of LAP (Ribeiro et al, 1999). To investigate the role of TSP-1 in mediating PAR<sub>1</sub>-dependent activation of TGF- $\beta$ , the effect of LSKL peptides and the scrambled peptide control SLLK on TFLLR-NH<sub>2</sub>-mediated TGF- $\beta$  activation was explored by using the tMLEC assay.

As seen in **Figure 3.38**, pre-incubation of pHALFs/tMLEC co-cultures with LSKL for 15 minutes blocked the TFLLR-NH<sub>2</sub>-induced increase in luciferase activity by 117% $\pm$  12.5. In contrast, the control peptide SLLK exerted no effect on TFLLR-NH<sub>2</sub>-induced TGF- $\beta$  activation. This study demonstrates that TFLLR-NH<sub>2</sub>-mediated activation of TGF- $\beta$  is TSP-1 dependent.



**Figure 3.38. Effect of TSP-1 blocking peptide on TFLLR-NH<sub>2</sub>-induced TGF- $\beta$  activation.**

Graph showing active TGF- $\beta$  levels post PAR<sub>1</sub> stimulation in the presence of LSKL and SLLK measured using a tMLEC-fibroblast co-culture system (1:1) where fibroblast-derived active TGF- $\beta$  is detected as an increase in luciferase activity by tMLEC expressing luciferase under the control of the TGF- $\beta$ -responsive PAI-1 promoter. \*and \*\*p<0.001

#### **3.4.7. FXa-induced $\alpha$ -SMA expression is dependent on TGF- $\beta$ activity**

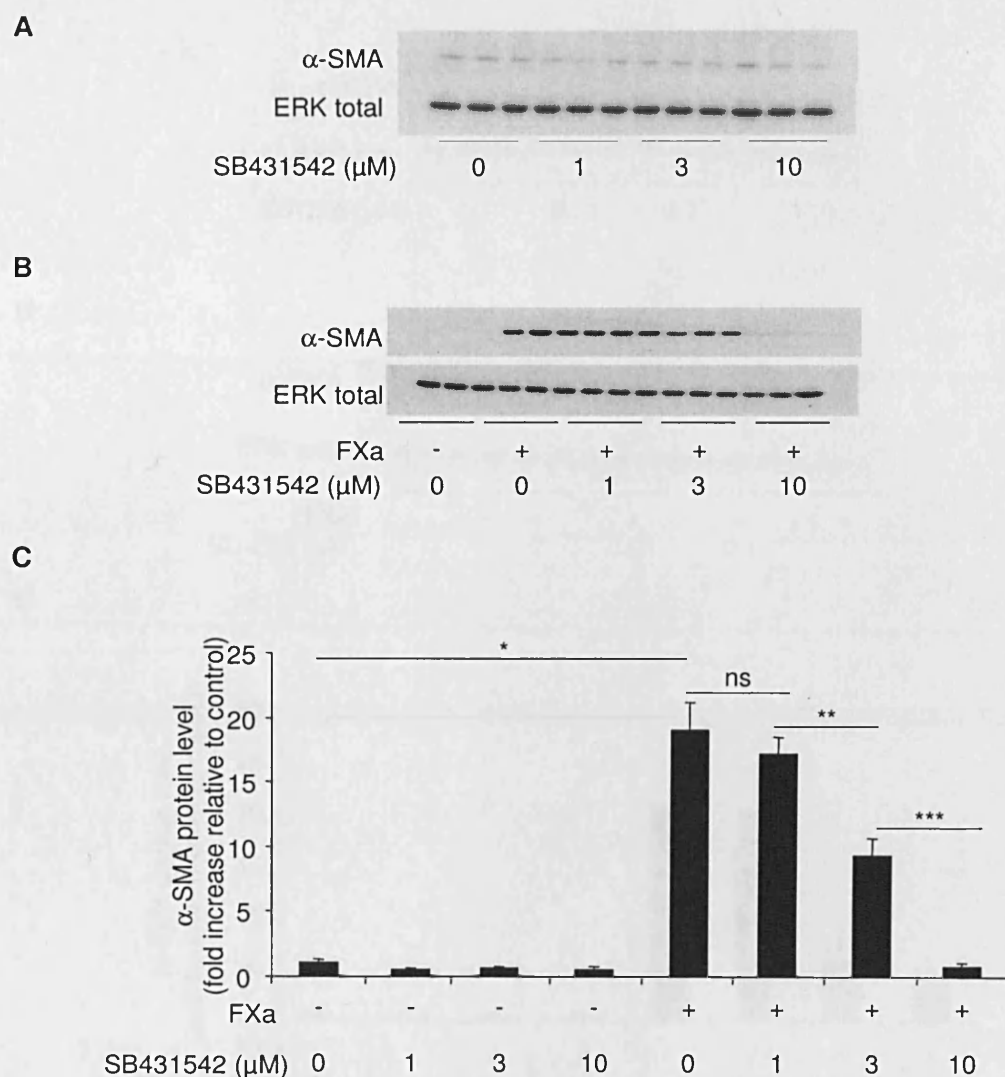
To answer the question of whether FXa-stimulated  $\alpha$ -SMA expression was dependent on TGF- $\beta$  activity, the influence of two pharmacologically unrelated selective small molecule inhibitors of the TGF- $\beta$  type 1 receptor kinase (ALK5) were investigated. Both compounds, SB431542 and SD-208 have previously been shown to inhibit TGF- $\beta$  induced myofibroblast differentiation (Mori et al, 2004; Kapoun et al, 2006).

Pre-incubation of pHALFs with SB431542 and SD-208 for 30 minutes prior to stimulation with FXa reduced  $\alpha$ -SMA protein levels in a concentration-dependent manner (**Figures 3.39. and 3.40.**). At the highest concentrations of SB431542 (10 $\mu$ M) and SD-208 (0.1 $\mu$ M),  $\alpha$ -SMA protein levels were blocked by about 95% and 99% respectively. At these concentrations, neither inhibitor influenced baseline expression of  $\alpha$ -SMA but fully blocked  $\alpha$ -SMA protein levels induced by 1ng/ml of exogenous TGF- $\beta$  (**Figures 3.41. and 3.42.**). These data confirm that FXa exerts its stimulatory effect on  $\alpha$ -SMA expression via a TGF- $\beta$ -dependent mechanism.

#### **3.4.8. PAR<sub>1</sub>, $\alpha$ -SMA and TSP-1 colocalise in human fibrotic lesions**

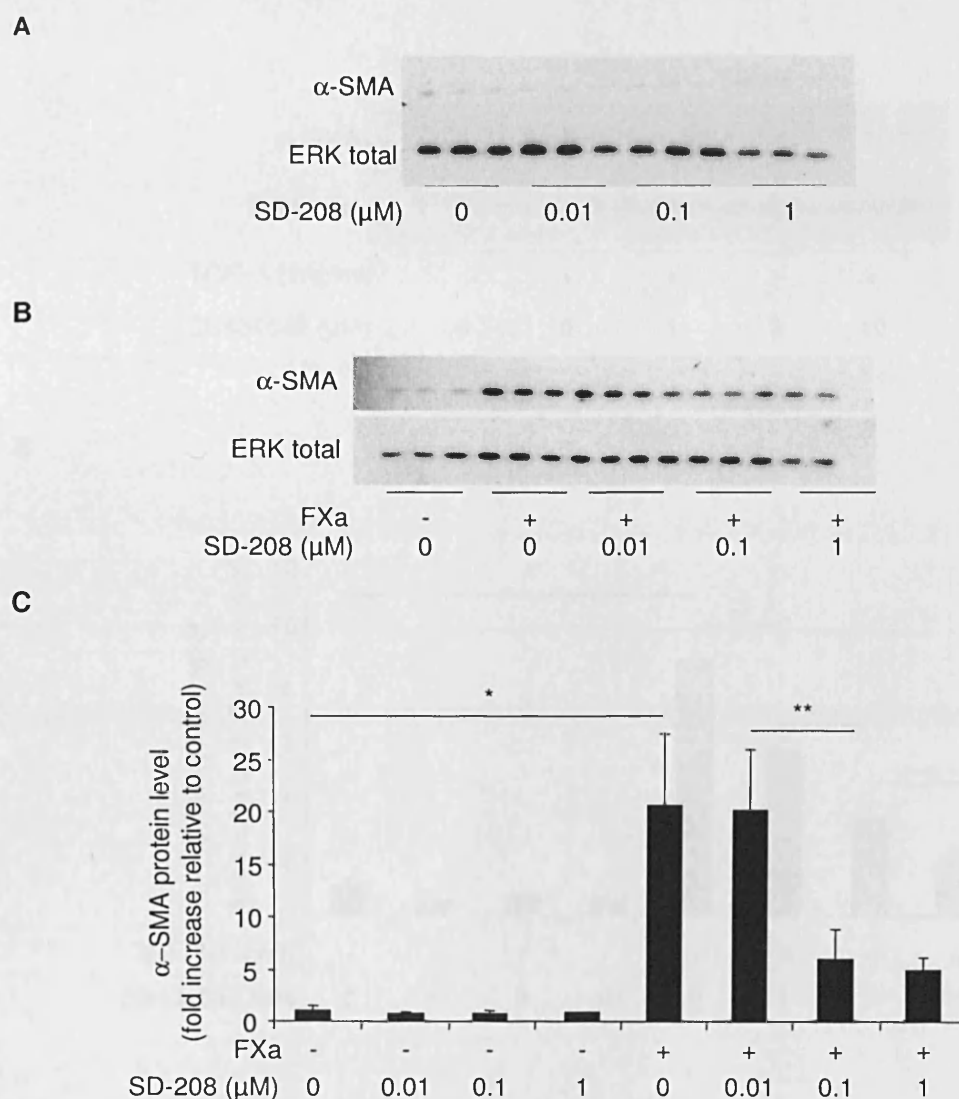
The data presented here support the conclusion that FXa is a potent inducer of fibroblast to myofibroblast differentiation via TSP-1-mediated activation of TGF- $\beta$ . To further explore the possibility that this mechanism might be operative in human fibrotic lung disease, PAR<sub>1</sub>, TSP-1 and  $\alpha$ -SMA immunoreactivity was assessed in serial sections from human IPF lung tissue (Brompton cohort).

**Figure 3.43. A-D** shows that all three proteins were highly expressed within the fibrotic foci of these lung sections. PAR<sub>1</sub> and  $\alpha$ -SMA were co-localised on numerous cells with a typical spindle-shaped myofibroblast appearance; whereas TSP-1 was localized to these cells as well as the matrix. The hyperplastic epithelium overlying fibrotic foci was completely negative for TSP-1.



**Figure 3.39. Effect of inhibition of ALK5 activity with SB431542 on FXa-induced  $\alpha$ -SMA expression.**

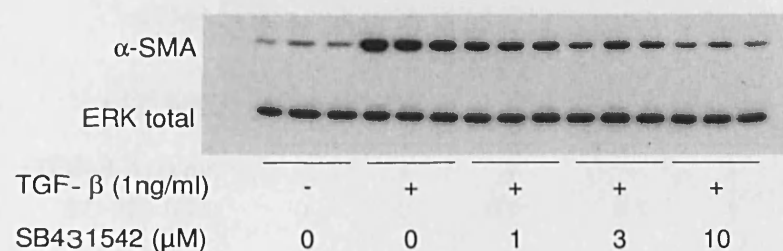
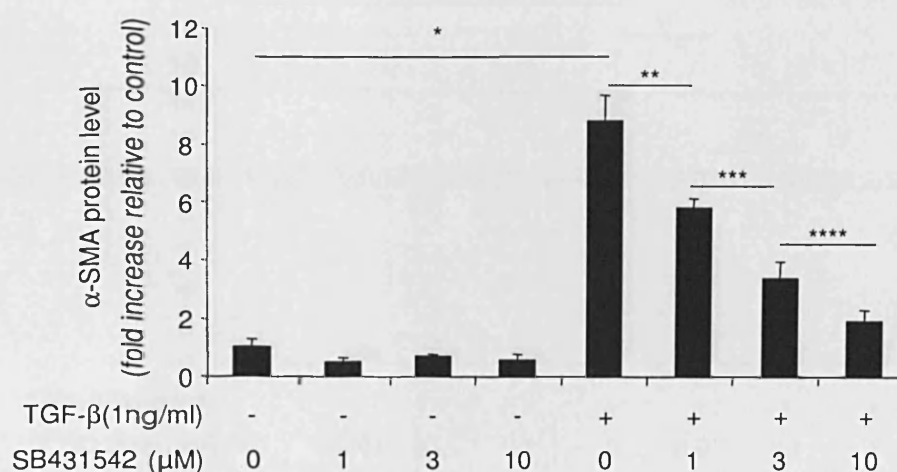
(A) SB431542 did not affect baseline  $\alpha$ -SMA protein levels at any concentration. (B) SB431542 attenuated FXa-induced  $\alpha$ -SMA protein levels in a concentration-dependent manner.  $\alpha$ -SMA was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. (C) The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Q94 blocked up to 95% of FXa induced  $\alpha$ -SMA protein levels at its highest concentration. The same control samples were used within the same experiment to allow for normalisation between blots. Representative experiment for three separate experiments performed. All data represented as mean fold increase over media control  $\pm$  s.d. \*and \*\*and \*\*\* $p < 0.001$



**Figure 3.40. Effect of inhibition of ALK5 activity with SD-208 on FXa-induced  $\alpha$ -SMA expression.**

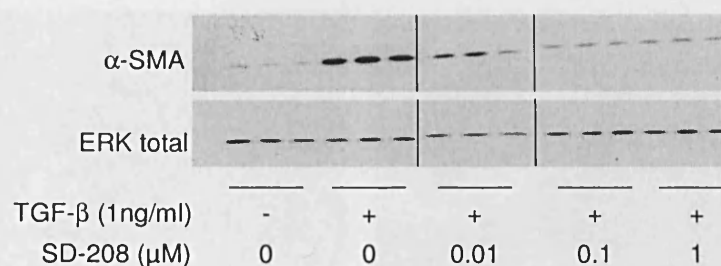
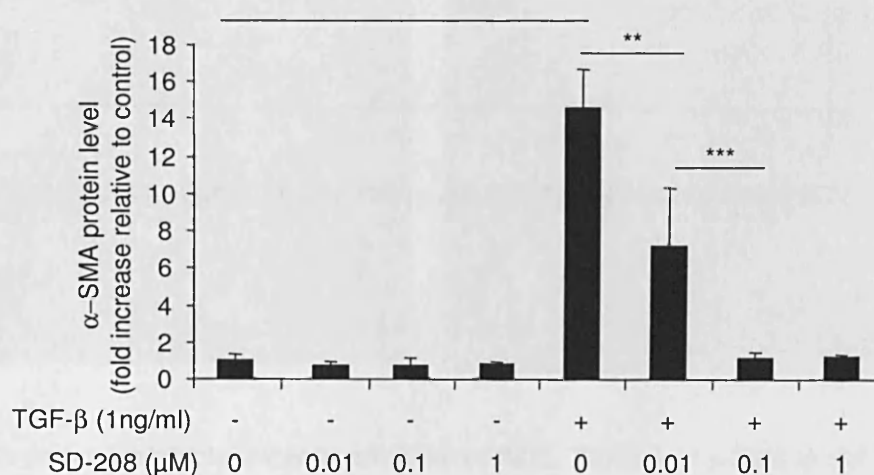
(A) SD-208 did not affect baseline  $\alpha$ -SMA protein levels at any concentration. (B) SD-208 attenuated FXa-induced  $\alpha$ -SMA protein levels in a concentration-dependent manner.  $\alpha$ -SMA was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobbed with total ERK2 as protein loading control. (C) The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. SD-208 blocked over 98% of FXa induced  $\alpha$ -SMA protein levels at its highest concentration. The same control samples were used within the same experiment to allow for normalisation between blots. Representative experiment for three separate experiments performed. All data represented as mean fold increase over media control  $\pm$  s.d. \* $p < 0.001$



**A****B**

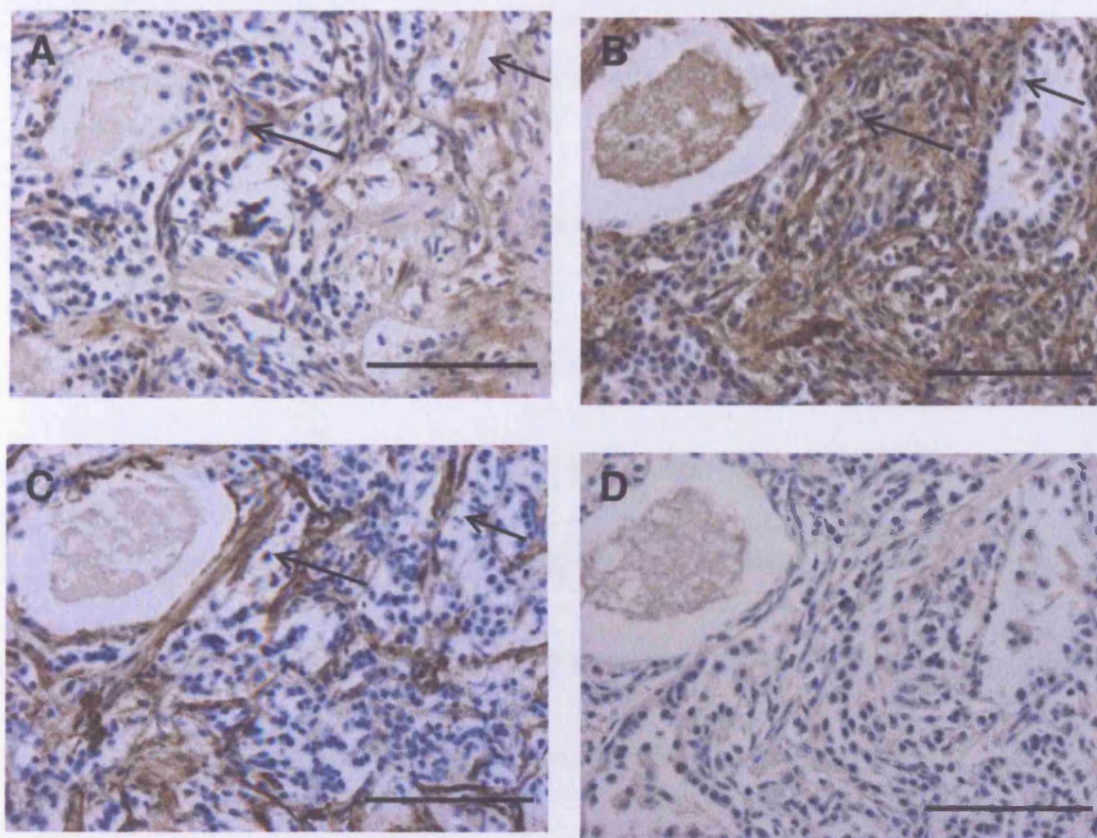
**Figure 3.41. Effect of inhibition of ALK5 activity with SB431542 on TGF-β-induced α-SMA expression.**

(A) SB431542 attenuated TGF-β-induced α-SMA protein levels in a concentration-dependent manner. α-SMA was detected using an anti-α-SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. (B) The relative increase in α-SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. The effect of SB431542 on baseline α-SMA protein is shown in **Figure 3.38 (A)**. The same control samples were used within the same experiment to allow for normalisation between blots. All data shown is representative of three separate experiments and shown as mean fold increase  $\pm$  s.d., based on three replicates in each experimental group \*and \*\*and\*\*\* $p < 0.001$ , \*\*\*\* $p = 0.021$

**A****B**

**Figure 3.42. Effect of inhibition of ALK5 activity with SD-208 on TGF-β-induced α-SMA expression.**

(A) SD-208 attenuated TGF-β-induced α-SMA protein levels in a concentration-dependent manner. α-SMA was detected using an anti-α-SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. Lines indicate that protein bands were not positioned directly adjacent to each other on the blot. (B) The relative increase in α-SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. Graph showing effect of SD-208 on baseline α-SMA protein is shown in **Figure 3.5 (A)**. The same control samples were used within the same experiment to allow for normalisation between blots. All data shown is representative of three separate experiments and shown as mean fold increase  $\pm$  s.d., based on three replicates in each experimental group \*and \*\*and\*\*\* $p < 0.001$



**Figure 3.43. Immunohistochemical localisation of PAR<sub>1</sub>, TSP-1 and  $\alpha$ -SMA in IPF**

Photomicrographs of representative 3 $\mu$ m serial histological sections showing immunoreactivity for PAR<sub>1</sub> (A), TSP-1 (B), and  $\alpha$ -SMA (C) in human IPF fibrotic foci. Spindle-shaped (myo) fibroblast-like cells co-localise for  $\alpha$ -SMA, PAR<sub>1</sub> and TSP-1 (black arrows). IgG isotype control section showed no apparent staining (D). Scale bars, 100 $\mu$ m.

---

**3.4.9. Summary**

The results described in this section examining the role of TGF- $\beta$  in FXa-mediated myofibroblast differentiation showed:

- FXa stimulates TGF- $\beta$  activity but does not regulate TGF- $\beta$  production at least at the mRNA level.
- The PAR<sub>1</sub>-activating peptide TFLLR-NH<sub>2</sub> induces activation of latent TGF- $\beta$
- FXa stimulates TSP-1 expression at the mRNA and protein level
- PAR<sub>1</sub>-mediated activation of latent TGF- $\beta$  is dependent on TSP-1
- FXa-induced  $\alpha$ -SMA expression is dependent on TGF- $\beta$  activity
- PAR<sub>1</sub>,  $\alpha$ -SMA and TSP-1 colocalise in human fibrotic lung tissue

In summary, these studies demonstrate that FXa is a potent inducer of the myofibroblast differentiation programme via the upregulation of TSP-1 and subsequent activation of latent TGF- $\beta$ .

### 3.5. Effect of the direct FXa inhibitor ZK 807834 on FXa-induced pro-fibrotic effects *in vitro* and *in vivo*

#### 3.5.1. Introduction

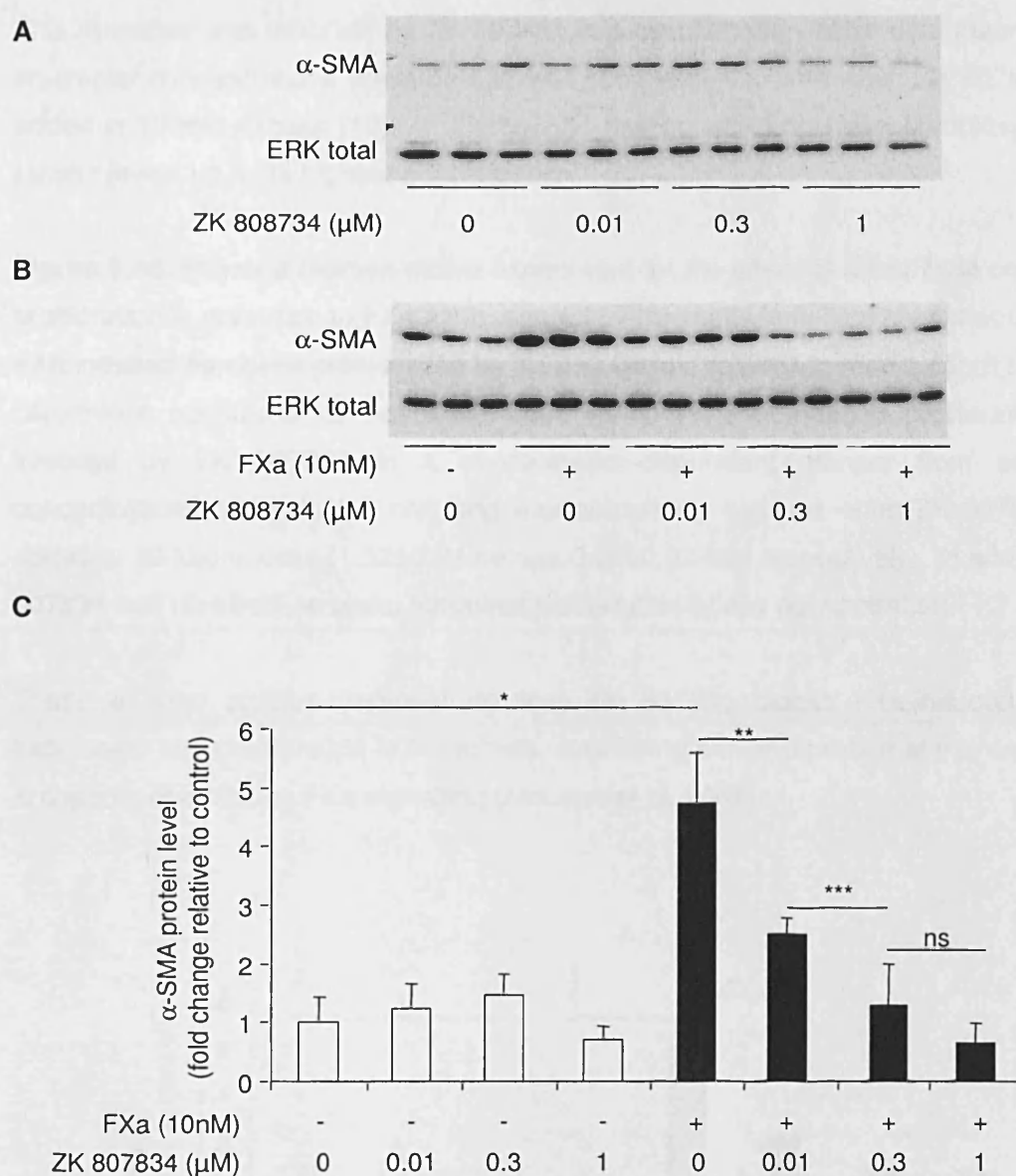
The final aim of this study was to examine the potential contribution of FXa to the development of lung fibrosis by evaluating its profibrotic role in the bleomycin model of lung injury and fibrosis- a model that has previously been shown to be dependent on PAR<sub>1</sub> signalling by our host laboratory(Howell et al, 2005). To this end the effect of the direct FXa inhibitor, ZK 807834 was assessed in this animal model. This compound is a direct inhibitor of FXa and can inactivate both free FXa and FXa that is complexed with either TF/FVIIa, or FVa (the prothrombinase complex) (Phillips et al, 1998). These FXa conformations are relevant in the extravascular space, in particular the potent TF/FVIIa/FXa kinase signalling complex (Ruf et al, 2003). ZK807834 was therefore chosen to give the best possible chance of attenuating FXa activity in the bleomycin model of lung injury and fibrosis.

#### 3.5.2. Effect of ZK 807834 on myofibroblast differentiation by FXa

The work in this thesis and previous work in our laboratory have demonstrated that FXa is a potent inducer of fibroblast to myofibroblast differentiation and proliferation (Blanc-Brude et al, 2005). In order to confirm the efficacy of ZK 807834 in blocking FXa signalling, the effect of this compound was initially evaluated against these FXa-induced fibroblast responses *in vitro*. Myofibroblast differentiation was measured by western blotting for  $\alpha$ -SMA protein as described in Results **Section 3.2.** Fibroblast proliferation was measured using the methylene blue assay, a spectrophotometric assay based on uptake and subsequent elution of the dye methylene blue, according to the method described by (Oliver et al, 1989) (see **Methods Section 2.22.**).

**Figure 3.44.** shows a representative experiment for the effect of ZK 807834 on FXa-induced  $\alpha$ -SMA expression. Prior to addition to cell cultures, ZK 807834 was preincubated at a range of concentrations (10nM-1 $\mu$ M) with a single dose of FXa (10nM) in serum- free DMEM for 60 minutes at 37 °C. To examine the effects of FXa alone, the protease was similarly preincubated in serum-free DMEM supplemented with equal concentrations of the ZK 807834 diluent at equal pH. In the experiment presented, exposure of pHALFs to FXa for 36hours in serum-free conditions induced  $\alpha$ -SMA protein levels by 4.73-fold  $\pm$  0.87 relative to media control cells.





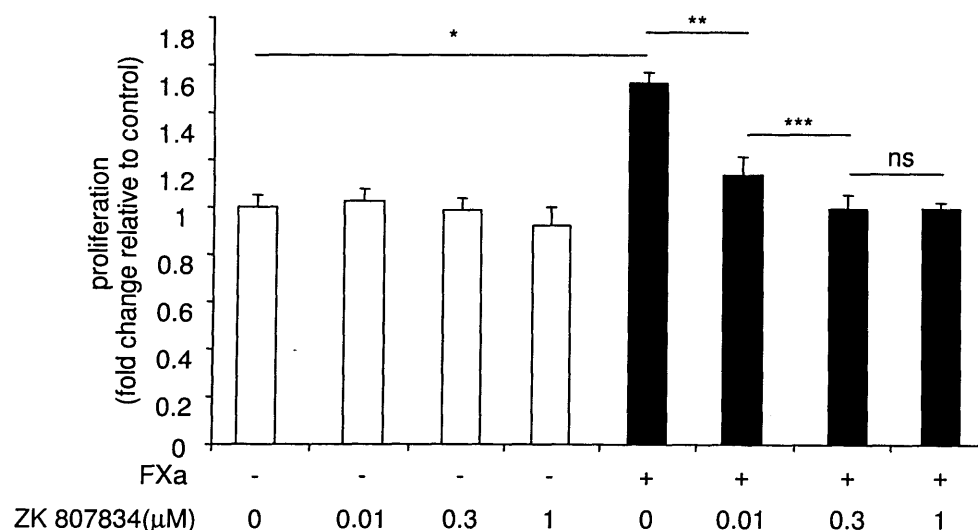
**Figure 3.44. Effect of ZK 808734 on FXa-induced  $\alpha$ -SMA expression**

(A) ZK 808734 had no effect on baseline  $\alpha$ -SMA protein levels. (B) ZK 808734 attenuated FXa-induced  $\alpha$ -SMA protein levels in a concentration-dependent manner.  $\alpha$ -SMA protein was detected using an anti- $\alpha$ -SMA antibody and blots were then stripped and reprobed with total ERK2 as protein loading control. (C) The relative increase in  $\alpha$ -SMA protein levels normalised to total ERK2 protein levels was determined by semi-quantitative densitometry. The same control samples were used within the same experiment to allow for normalisation between blots. All data shown is representative of three separate experiments and shown as mean fold increase  $\pm$  s.d., based on three replicates in each experimental group, \*and \*\* $p < 0.001$ , \*\*\* $p = 0.011$

This response was inhibited by ZK 807834 in a concentration-dependent manner from equimolar concentrations onwards and was completely blocked when ZK 807834 was added in 10-fold excess (10 $\mu$ M). ZK 807834 had no effect on basal fibroblast  $\alpha$ -SMA protein levels up to its highest concentration.

**Figure 3.45.** shows a representative experiment for the effect of ZK 807834 on pHALF proliferation in response to FXa as measured by the methylene blue colorimetric assay. FXa induced fibroblast proliferation by  $1.52 \pm 0.04$ -fold relative to media control cells, in serum-free conditions as assessed after 48 hours. FXa-induced proliferation was inhibited by ZK 807834 in a concentration-dependent manner from equimolar concentrations onwards (10 nM) and was completely blocked when ZK 807834 was added in 10-fold excess ( $1.52 \pm 0.04$ -versus  $0.99 \pm 0.02$ -fold respectively). In addition, ZK 807834 had no effect on basal fibroblast proliferation at any concentration.

These *in vitro* studies demonstrate that ZK 807834 blocks FXa-induced  $\alpha$ -SMA expression and proliferation in fibroblasts, confirming earlier reports that this compound is capable of inhibiting FXa signalling (McLean et al, 2001).



**Figure 3.45. Effect of ZK 807834 on FXa-induced fibroblast proliferation in vitro**

Figure shows the effect of the selective FXa inhibitor ZK 807834 on FXa-induced fibroblast proliferation as measured using the methylene blue colorimetric assay. All data shown is representative of three separate experiments and expressed as mean fold increase  $\pm$  s.d., based on six replicates in each experimental group \*and \*\*and \*\*\* $p < 0.0001$

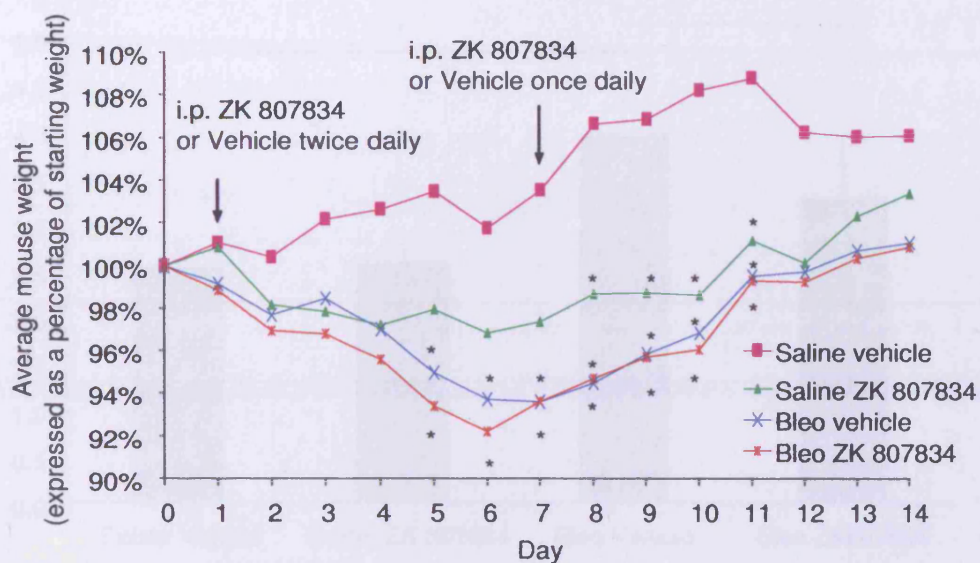
### 3.5.3. Effect of ZK 807834 on bleomycin-induced lung injury and fibrosis

Having confirmed the efficacy of ZK 807834 in blocking FXa-mediated fibroblast responses *in vitro*, the effect of this compound was evaluated in the bleomycin model of lung injury and fibrosis. Given the chronic nature of FXa generation following bleomycin administration, a daily dosing regime was chosen based on the pharmacokinetic data provided by Berlex, indicating that a circulating concentration of ZK 807834 above 2 $\mu$ M was sufficient to double the prothrombin time in C57Bl/6J mice. Twenty-four hours post-bleomycin, administration of ZK 807834 (50mg/kg in acidified saline) or vehicle was started, by intraperitoneal injection twice a day in the first week and once a day in the second week until the end of the experiment.

**Figure 3.46.** shows the effect of intraperitoneal ZK 807834 on mouse body weight following administration of bleomycin or saline over a 14 day period. All animals given bleomycin showed a characteristic decrease in body weight compared to control animals treated with saline from days 5 onwards. In addition, animals in the saline/ZK 807834 group had a significant decrease in body weight compared to mice in the saline/vehicle group at days 8 to 11. In terms of animal survival, three mice in the saline/ZK807834 group (originally n=6) and three mice in the bleomycin/ZK807834 group (originally n=9) were sacrificed due to intraperitoneal hemorrhage. This, however, was thought to arise from the tissue damage caused by the administration of multiple intraperitoneal injections.

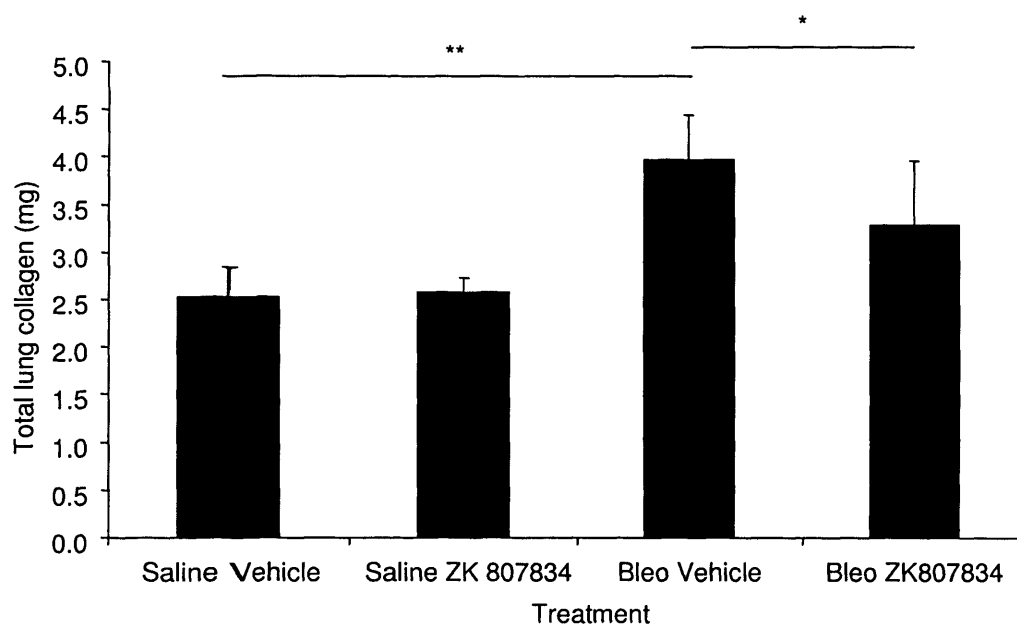
**Figure 3.47.** shows that daily i.p. injection of mice with this compound from 24hrs following bleomycin injury, led to a significant attenuation in lung collagen accumulation at 14 days. Total lung collagen was increased by 60% in bleomycin-injured mice compared with saline-instilled control animals. This increase was significantly reduced to only 27% in bleomycin-injured mice given ZK 807834. These data confirm the importance of FXa in driving the fibrotic response in this mouse model.





**Figure 3.46. Effect of ZK 807834 on mouse body weight in bleomycin-induced lung injury and fibrosis**

Figure shows the effect of ZK 807834 (50mg/kg) on mouse body weight following a single instillation of bleomycin (1mg/kg body weight) by oropharyngeal administration, or saline. Results are expressed as mean change in body weight as a percentage of the starting weight, \* $p < 0.001$ .



**Figure 3.47. Effect of ZK 807834 on lung collagen accumulation in bleomycin-induced lung injury and fibrosis**

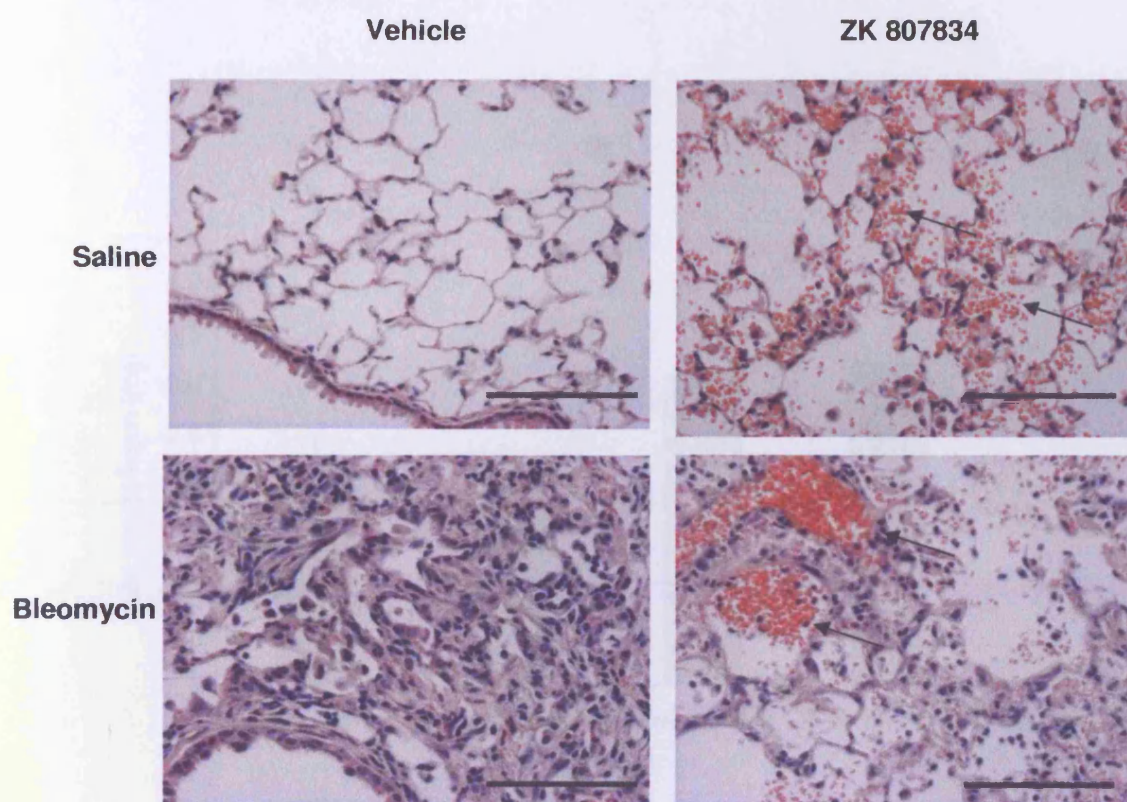
Figure shows the effect of daily i.p injections of ZK 807834 on lung collagen accumulation 14 days following a single oropharyngeal instillation of bleomycin (1mg/kg). Lung collagen was measured by reverse phase HPLC quantitation of lung hydroxyproline in acid hydrolysates of pulverized lung. Total amount of collagen in each lung was calculated assuming that lung collagen contains 12.2% w/w hydroxyproline (see Method Section 2.9.) Data represent the mean  $\pm$  s.e.m of values obtained in groups of six (saline vehicle and bleomycin ZK 807834), three (saline ZK 807834) or seven (bleomycin vehicle) mice. \* $p=0.021$ , \*\* $p<0.001$

#### **3.5.4. Effect of oropharyngeal instillation of ZK 807834 in bleomycin-induced lung injury and fibrosis**

In order to assess the effect of localised inhibition of FXa on bleomycin-induced lung injury and fibrosis a site-targeted route of administration in the form of oropharyngeal instillations of ZK 807834 was chosen. It was felt that this route of administration would allow targeting of extravascular FXa activity whilst minimising systemic effects of anticoagulation.

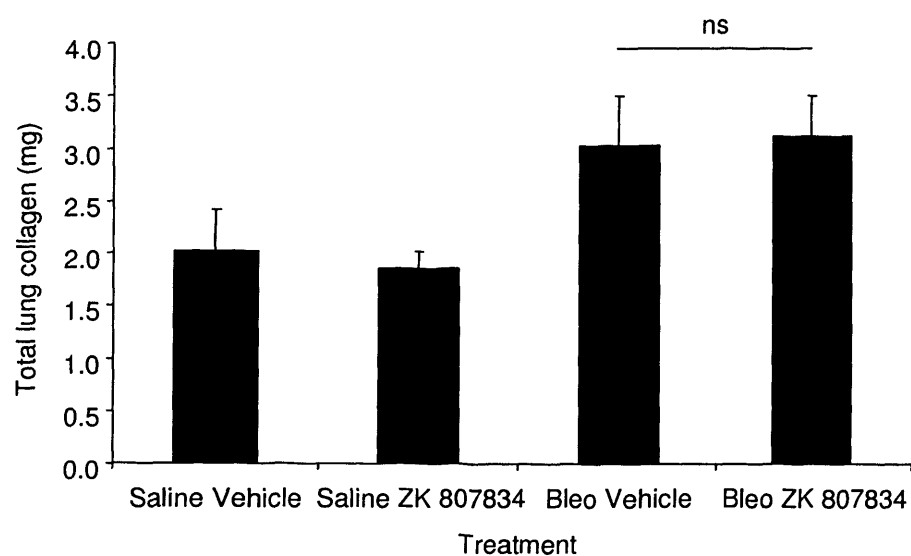
Twenty-four hours post-bleomycin (single dose 1mg/kg), administration of ZK 807834 (50mg/kg in acidified saline, at the same dose that significantly attenuated lung collagen accumulation with multiple i.p. injections) or vehicle was started, by once daily oropharyngeal instillation in 50µl of acidified saline at pH 5.

**Figure 3.48.** shows representative images of histological sections of mice lungs treated with or without ZK 807834 taken from animals. About 70% of animals in the ZK 807834/bleomycin and ZK 807834/saline group died of extensive intrapulmonary haemorrhage (**B and D**). As a result, in the next set of experiments, a 5-fold lower concentration of ZK 807834 was administered (10mg/kg) by daily oropharyngeal instillation. **Figure 3.49.** shows the effect on lung collagen accumulation at 14 days. ZK 807834 administered oropharyngeally failed to attenuate the increase in total lung collagen, suggesting that this dose of anticoagulation may not have been sufficiently high to block FXa signalling.



**Figure 3.48. The effect of oropharyngeal instillation of ZK 807834 on lung histopathology in bleomycin-induced lung injury and fibrosis**

Representative images showing histological sections of lung tissue taken from mice challenged with bleomycin or saline and treated daily ZK 807834 or vehicle, stained with haematoxylin and eosin to assess histopathological changes. Lung architecture was normal in mice challenged with saline. Extensive patchy fibrotic foci were detected in the lungs of mice challenge with bleomycin at 14 days. Extensive haemorrhagic lesions (black arrows) were observed in the lungs of mice treated with ZK 807834 both in saline and bleomycin group. Histopathological pattern was assessed by three independent reviewers (n= 3 mice in ZK 807834 and saline group, n=3 mice in ZK 807834 and bleomycin group, n=6 in saline and vehicle and bleomycin and vehicle group). Scale bars 100µm.



**Figure 3.49. Effect of administration of ZK 807834 by oropharyngeal instillation in bleomycin-induced lung injury and fibrosis**

Figure shows the effect of daily oropharyngeal instillation of ZK 807834 (10mg/kg) on lung collagen accumulation 14 days following a single oropharyngeal instillation of bleomycin (1mg/kg). Lung collagen was measured by reverse phase HPLC quantitation of lung hydroxyproline in acid hydrolysates of pulverized lung. Total amount of collagen in each lung was calculated assuming that lung collagen contains 12.2% w/w hydroxyproline (see Methods Section 2.9.) Data represent the mean  $\pm$  s.e.m of values obtained in six mice per group.

---

**3.5.5. Summary**

The results described in this chapter examine the effect of ZK 807834 on bleomycin-induced lung injury and fibrosis

- The direct FXa inhibitor ZK 807834 significantly blocks FXa-induced fibroblast to myofibroblast differentiation and proliferation in a concentration-dependent manner.
- Intraperitoneal injection of the direct FXa inhibitor ZK 807834 significantly attenuated lung collagen accumulation in bleomycin-induced lung injury and fibrosis, at 14 days.
- Oropharyngeal administration of the direct FXa inhibitor ZK 807834, caused extensive hemorrhagic lesions in bleomycin-induced lung injury and fibrosis, at 14 days.
- Oropharyngeal administration of the direct FXa inhibitor ZK 807834, administered at a 5-fold lower dose had no effect on lung collagen accumulation in the bleomycin-induced lung injury and fibrosis, at 14 days.

---

## Chapter 4: Discussion

### Overview

Differentiation of fibroblasts into highly synthetic and contractile,  $\alpha$ -SMA expressing myofibroblasts plays a central role in the fibrotic response to lung injury. A number of human fibroproliferative disorders, including IPF are associated with uncontrolled activation of the coagulation cascade, with extravascular expression of TF leading to the activation of coagulation proteinases including FVII, FX and prothrombin (Hernandez Rodriguez et al, 1995; Idell, 2003; Gunther et al, 2003). Coagulation proteinases induce multiple pro-inflammatory and pro-fibrotic cellular effects via the activation of their major signalling receptors the PARs, including PAR<sub>1</sub>. *In vivo* PAR<sub>1</sub> deficient- mice are partially protected from acute inflammation and subsequent chronic fibrosis following bleomycin-induced lung injury (Howell et al, 2005).

Until recently, coagulation zymogens were thought to be mainly derived from the circulation and locally activated in response to tissue injury. However, global expression profiling studies performed in our laboratory revealed that the zymogen FX is locally upregulated in the murine lung in response to bleomycin-induced lung injury suggesting that this proteinase may play a central role in promoting fibrogenesis. This thesis therefore examined the hypothesis that **FX/FXa is locally expressed in the fibrotic lung and contributes to the development of pulmonary fibrosis by influencing fibroblast function.**

To address this hypothesis, the initial experiments of this thesis employed real time RT-PCR analysis and immunohistochemistry to examine FX gene and protein expression in the bleomycin model of lung injury and fibrosis. These studies confirmed that FX is locally upregulated following experimentally-induced lung injury. In terms of human disease relevance, this finding was confirmed by laser-capture microdissection of alveolar septae and immunohistochemistry of lung biopsy material from patients with IPF across three separate patient cohorts. These studies further identified the alveolar and bronchial epithelium as a prominent cellular source of FX in the fibrotic lung. In addition, this thesis reports for the first time that FXa is a potent inducer of the myofibroblast differentiation programme. PAR<sub>1</sub> was identified as the major FXa signalling receptor involved in mediating this response via the transcriptional upregulation of the matricellular protein thrombospondin-1 (TSP-1) and the subsequent activation of the potent pro-fibrotic mediator, TGF- $\beta$ <sub>1</sub>. Finally, the role of FXa in the development of lung fibrosis *in vivo* was examined using a direct inhibitor of FXa in the

bleomycin model of lung injury and fibrosis, ZK 807834. These studies show that lung fibrosis in mice treated daily with this inhibitor from 24 hours after the initial insult was significantly reduced.

In summary, these data support the notion that FXa, a central proteinase of the coagulation pathway, is locally produced in the injured lung and represents a novel pathogenic factor that drives the fibrotic response to lung injury. The following sections will discuss these findings and their implications in more detail.



## 4.1. Local FX generation in the lung

### 4.1.1. *FX is expressed locally in the lung and is upregulated in fibrosis*

The observation that the gene encoding FX is expressed and upregulated in the injured lung was initially made by Drs Scotton and Lee by microarray analysis of lung RNA from bleomycin-injured mice. Although this was not the first study to publish transcriptional profiling data in this model, the data produced was extensive in that the Affymetrix genechips contained probe sets representing approximately 14000 genes. As expected, for the fibrotic phase of the response to bleomycin, the upregulation of genes encoding proteins such as collagen I, III, IV and fibronectin was identified. Gene ontology data mining classified these genes into gene families which are implicated in fibrosis, such as “mesenchymal cell differentiation” and found that in the group of genes classified as genes involved in the ‘wound healing response’, FX was one of the most highly upregulated genes post-bleomycin injury.

Microarray analysis has revolutionized the field of molecular biology, allowing for the detection of the expression pattern of thousands of genes and their interactions simultaneously. However, this novel technology is a large-scale approach which therefore requires quantitative and qualitative validation. In addition, due to financial limitations, microarray studies are often performed with a limited number of replicates, such as in our study which examined 3 mice per experimental group only. The initial experiments of this thesis therefore employed quantitative real-time RT-PCR to investigate the reproducibility of the results obtained by microarray analysis. To this end, FX gene expression of whole lung homogenates was measured in two mouse models of bleomycin-induced lung injury and fibrosis. The first was induced by oropharyngeal (OP) bleomycin instillation whereas the second was induced by aerosolised administration (AS) of bleomycin (see Methods **Section 2.6.**) for more detailed description of the individual models). These methods of administration were preferentially chosen over the traditional method of intratracheal (IT) challenge of bleomycin which was used for all microarray studies. Whilst all models appear to have identical increases in total lung collagen 14 days post-bleomycin challenge, the distribution of fibrotic foci as observed histologically is different in the OP and AS models compared to the IT model. Localised lesions which do not appear to extend into the distal parenchyma can be observed following IT instillation; in contrast, OP and AS instillation results in a more diffuse pathology with multiple fibrotic foci distributed throughout the lung parenchyma, reminiscent of human fibrotic lung disease. In addition, these models were found to be more practical and less distressing to the mice

because they do not require deep anaesthesia and surgery compared to the traditional route of administration.

The studies performed in our and Dr Eickelberg's laboratories confirmed that FX was expressed in the murine lung at the mRNA level and was highly upregulated in response to bleomycin injury. Subsequent immunohistochemical analysis of tissue sections demonstrated strong immunoreactivity for FX/FXa associated with the bronchial and alveolar epithelium, as well as macrophages and spindle-shaped (myo)fibroblasts within fibrotic foci. In terms of the potential relevance of these findings to human fibrotic lung disease, FX was also expressed in lung biopsy transplant tissue from patients with pulmonary fibrosis by real time RT-PCR. Analysis of FX mRNA levels in total lung homogenates did not reveal upregulation of FX mRNA levels in human fibrotic lung tissue. However, laser-capture microdissection of alveolar septae demonstrated marked upregulation of FX mRNA levels in IPF patients compared with control lung. Immunohistochemical analysis of FX/FXa protein localisation further showed consistent immunoreactivity of FX/FXa associated with the bronchial and alveolar epithelium, macrophages and fibrotic foci, in biopsy tissue samples from 18 patients with UIP which were derived from independent cohorts in the USA and the UK. In summary, these results provide compelling evidence that the zymogen FX is made locally in the fibrotic lung.

Until recently, it was generally held that coagulation zymogens are largely synthesised in the liver (Greenberg et al, 1995; Hung and High, 1996). Although FX expression at the mRNA level has been reported in other tissues (Wilcox et al, 2003), the expression levels are very low compared with those in the liver. In contrast, several *in vitro* studies suggest that coagulation zymogen FVII may be synthesised extrahepatically. For example, FVII expression has been documented in monocytes/ macrophages isolated from patient lungs with interstitial lung disease (Chapman, Jr. et al, 1985) and following LPS stimulation (Tsao et al, 1984). Similarly, ovarian cancer cells are also capable of synthesising FVII *in vitro* (Koizume et al, 2006). *In vivo*, FVII mRNA has been detected in human smooth muscle cells and macrophages in atherosclerotic lesions by *in situ* hybridisation (Wilcox et al, 2003). In contrast, evidence for an extrahepatic source of FX is inconclusive. A recent study published during the course of this PhD suggested that infiltrating macrophages may express FX in an experimental model of asthma (Shinagawa et al, 2007). However, this study showed that local resident lung cells did not synthesise the zymogen. The work presented in this thesis is therefore the first to report that FX is synthesised locally in the murine and human lung and that its

expression is increased in experimental and human fibrotic lung disease. This finding supports the notion that in pathological states, other organs besides the liver are capable of expressing coagulation zymogens such as FX.

#### **4.1.2. Alveolar epithelial cells produce FX**

Having made the observation that FX expression was increased in the fibrotic lung, studies were undertaken to identify the cellular source responsible for the synthesis of this zymogen. Initial observations by immunohistochemistry of tissue sections taken from saline and bleomycin-treated mouse lungs showed a much localised staining pattern of FX/FXa which was mainly associated with bronchial epithelial cells in saline-treated animals and with the bronchial and alveolar epithelium, as well as macrophages and myofibroblasts within fibrotic foci in bleomycin-injured lungs. This immunohistochemical pattern was found to be identical for biopsy tissue samples from patients with UIP which were derived from independent cohorts in the USA (n=18) and the UK (n=6). Although these immunohistochemical studies did not necessarily reveal which cells are responsible for the production of FX, but rather where it might be accumulating, the staining pattern was particularly cell-associated in alveolar epithelial cells overlying fibrotic regions, suggesting that these cells may represent a local source of FX production.

To answer this question more precisely, a recently developed laser-capture microdissection technique was applied to human donor and IPF lung tissue, in order to specifically sample the alveolar epithelial cell population (alveolar septae) from the alveolar compartment. Subsequent real-time RT-PCR was used for quantitative analysis of gene expression. Compared with other methods of gene expression analysis such as *in situ* hybridisation, this method allows quantification of mRNA changes in a sensitive and reliable manner (Fink et al, 1998; Fink et al, 2000b; Fink et al, 2000a). Prior to laser-capture microdissection, alveolar epithelial cells were identified by cell-specific immunostaining allowing for a more precise identification of type II alveolar epithelial cells compared to conventional identification by morphological inspection only (Fink et al, 2000a; Fink et al, 2000b). These studies revealed that FX gene expression was highly upregulated (5-fold induction) in alveolar septae from patients with IPF compared to donor lung. Further *in vitro* analysis of FX expression confirmed these observations, as alveolar and bronchial epithelial cell lines in culture expressed FX at the mRNA and at the protein levels. These data suggest that alveolar epithelial cells represent a prominent source of FX expression/production in human fibrotic lung disease. These studies lend further support to the notion that alveolar

epithelial cells may represent a prominent source of pro-inflammatory and pro-fibrotic mediators and thus play a key role in driving the fibrotic response to lung injury (Kasper and Haroske, 1996; Selman and Pardo, 2006).

Of interest, the upregulation in FX expression was not detectable using mRNA derived from total lung homogenates of the same donor and IPF lungs as used for laser capture microdissection studies. This result contrasts the findings obtained in the murine model of lung injury and fibrosis where a significant increase in FX expression was observed in whole lung homogenates. The difference in these findings may be explained by the difference in stages of disease pathology between both murine and human samples and by the heterogeneity of the human samples. In contrast to the murine samples which were all collected at single time points and from a genetically identical group of mice, human samples are much more varied, both in terms of the timing of the biopsy and the mixed genetic background of the patient groups so that a larger number of patient samples may be needed. Furthermore, the signal for FX expression may be diluted out by sampling total lung tissue so that the signal to noise ratio is too low when sampling total lung homogenates. In contrast, a statistically robust increase in FX gene expression was clearly discernable for IPF patients (n=5) when sampling the lung epithelium by laser-capture microdissection.

Analysis of fibroblasts isolated and then cultured from mouse lungs post-bleomycin injury showed a significant upregulation in FX mRNA levels compared to fibroblasts grown from saline-treated lungs. Although this finding was not replicated in human cultured fibroblasts, as mentioned above, samples used in this study only provide a single snapshot of the whole disease process in a heterogeneous group of patients at various stages of disease, so that an increase in FX gene expression may have been missed. Evidence that fibroblasts represent another prominent source of FX was provided by immunohistochemical studies which showed that myo(fibroblasts) in fibrotic foci stained positively for FX/FXa in human fibrotic lung samples. Further studies are clearly needed, possibly involving microdissected fibroblast samples as these will be more representative of the cell gene expression pattern *in vivo* than cells isolated from tissue and subsequently cultured *in vitro* and may thus have altered their expression pattern in culture. The observations that fibroblasts may synthesise FX are particularly interesting in light of the important role fibroblasts play as effector cells of coagulation signalling (Chambers et al, 1998; Chambers et al, 2000), which may thus represent an autocrine loop for FXa-driven effects.

#### **4.1.3. The lung is capable of generating the mature form of FX**

To investigate if cultured alveolar epithelial cells are capable of producing the mature form of FX, this thesis compared FX protein derived from alveolar epithelial cells to that produced by hepatocytes by western blotting. In hepatocytes, the newly synthesised single-chain FX precursor translocates to the endoplasmic reticulum as a pre-pro-protein, where the prepeptide signal is cleaved by a signal peptidase (Wallin and Turner, 1990). The remaining propeptide directs subsequent  $\gamma$ -carboxylation by the vitamin K-dependant  $\gamma$ -carboxylase which converts 10-12 glutamic acid residues in the N-terminus part of the protein into  $\gamma$ -carboxyglutamic acid (Gla) residues, an essential modification to ensure optimal activation of the zymogen once secreted (Furie and Furie, 1988). Finally, the single chain protein is cleaved into a double chain by an as yet unknown mechanism (Stanton and Wallin, 1992); Consistent with this mode of synthesis, the protein produced by these epithelial cell lines migrated as three bands of 74, 70 and 42 kDa, corresponding to the two single chain precursors and the heavy chain of the mature protein, respectively and in an identical fashion to that observed for the hepatocyte cell line HepG2 (Furie and Furie, 1988). In addition, the band pattern on western blotting of alveolar epithelial cells was consistent with the posttranslational processing observed for hepatocytes (Stanton and Wallin, 1992), which are classically viewed as the main cellular source of this zymogen. Interestingly, genechip studies performed in our laboratory further revealed that the enzymes  $\gamma$ -carboxylase (essential for  $\gamma$ -carboxylation of the zymogen) (Furie and Furie, 1988) and Vitamin K epoxide reductase (essential for the reduction of Vitamin K after it has been oxidized in the carboxylation of glutamic acid (Rost et al, 2004)) are expressed in the lung (data generated by Dr Scotton). Taken together, these data led to the conclusion that the lung epithelium has the capacity to synthesize the mature two-chain FX zymogen and represents an important cellular source of this zymogen in the fibrotic lung.

#### **4.1.4. Potential mechanisms leading to increased expression of FX in pulmonary fibrosis**

To begin to unravel the possible mechanisms that mediate local FX expression in the injured lung, initial *in vitro* experiments were performed to explore the influence of different stimuli on FX expression by alveolar epithelial cells. These experiments were performed on the epithelial cell line A549, which despite its pathological origin expresses all of the important markers of this cell type and is widely accepted and used as a model cell to study alveolar epithelial cell biology (Lieber et al, 1976). Initial experiments tested the effect of TGF- $\beta_1$  and TNF- $\alpha$  on FX expression. Both cytokines have been implicated in the development and progression of pulmonary fibrosis (see

Introduction **Section 1.3.**) and have previously been shown to upregulate TF expression by fibroblasts and epithelial cells (Idell et al, 1994). However, no stimulus-dependent variation in FX mRNA expression was observed with these mediators. In contrast, *in vitro* stimulation of A549 cells with bleomycin increased FX mRNA levels in a time-dependent manner at concentrations of the compound that have previously been shown to induce the release of pro-inflammatory mediators including CCL-2 by these cells (Sato et al, 1999). Bleomycin is a highly toxic molecule which is widely used to induce pulmonary fibrosis in rodents (reviewed in (Chua et al, 2005)) but also causes pulmonary fibrosis in patients receiving bleomycin as a chemotherapeutic agent for certain neoplasms (Carver et al, 2007). The exact mechanism by which bleomycin influences cell function and often causes cell death remains unclear however it has been proposed that the production of reactive oxygen species (ROS) by way of a ferrous ion-molecular oxygen-mediated mechanism which may directly lead to DNA strand break may play a role (Manoury et al, 2005). There is increasing evidence that oxidative stress, defined as an imbalance between oxidants and antioxidants may contribute to the progression of fibrotic lung disease (Lenz et al, 1996;Rottoli et al, 2005). For example, patients with IPF have been shown to have a redox imbalance with higher levels of oxidants (such as carbonylated proteins) and extracellular glutathione deficiency (Lenz et al, 1996;Behr et al, 2002). Reactive oxygen species are often secreted by inflammatory cells and fibroblasts and are thought to directly contribute to the pathogenesis of pulmonary fibrosis by mediating epithelial cell injury (Behr et al, 2002;Lenz et al, 2004). Increased levels of ROS correlate with the degree of epithelial cell injury and importantly a recent trial examining the effect of the antioxidant *N*-acetylcysteine showed a beneficial effect in slowing the progression of this disease (Kinnula et al, 2005). ROS mediated stress on alveolar epithelial cells may therefore represent a potential mechanism by which FX expression is induced in the fibrotic lung.

#### **4.1.5. Activation of FX in the extravascular space**

The work presented in this thesis supports the view that locally produced FX may be activated locally in the extravascular space. Activation of the zymogen FX can be catalysed by either FIXa/FVIIIa or by TF/FVIIa enzyme-cofactor complexes which both generate the identical FXa product, although it is generally believed that the TF/FVIIa complex plays a major role in the activation of FXa in the extravascular space, leading to the initiation of the extrinsic coagulation pathway (reviewed in (Idell, 2003), also see Introduction **Section 1.7.1**). Previous studies have shown that TF is constitutively expressed by alveolar macrophages, epithelial cells and fibroblasts in the normal

murine lung (Olman et al, 1995). Importantly, these cells upregulate the expression of TF following tissue injury and inflammation and both fibroblasts and macrophages express higher levels of TF in developing fibroproliferative lesions of mouse lungs challenged with bleomycin (Olman et al, 1995). In terms of human fibrotic lung disease, TF is expressed by epithelial cells overlying fibrotic foci and to some extent by alveolar macrophages, in close association with fibrin deposits in the lungs of patients with IPF and systemic sclerosis (Imokawa et al, 1997) and in pulmonary fibrosis associated with chronic lung disease of prematurity (Dik et al, 2003b). Similarly, alveolar macrophages from patients with fibrotic lung disease have been shown to express FVII *in vitro* (Chapman, Jr. et al, 1985). In studies presented in this thesis FX/FXa protein was immunolocalised to the bronchial and alveolar epithelium, fibroblasts and macrophages suggesting that FX localises to cells which express TF/FVII where FX is likely to be subsequently activated by the tenase complex.

Further evidence that locally generated FX is converted to FXa can be inferred from the timing for the increase in FX/FXa staining in the lungs of mice challenged with bleomycin. Bleomycin-induced lung injury is associated with epithelial and importantly endothelial damage causing an increase in endothelial permeability and vascular leak of plasma proteins into the alveolar space. This process is thought to peak early in the bleomycin model when inflammation is present (Howell et al, 2001). Whilst FX/FXa immunostaining was detected 7 days post-bleomycin challenge, the signal was dramatically increased in the later stages (14 days post-bleomycin instillation). Although, this does not exclude that FX may be leaking into the lung parenchyma as a result of microvascular leak, it is likely that local production of FX may play a major contributing role in the signal observed at 14 days. Furthermore, increased FXa activity was measured in the BALF obtained from mice challenged with bleomycin compared to saline-instilled animals. BALF is thought to predominantly sample the alveolar epithelium, thus suggesting that alveolar epithelial cell-derived FX may contribute to this pool of FX thus providing the extravascular substrate for the tenase complex, leading to local generation of FXa.

The significance of vascular leak in the pathogenesis of human fibrotic lung disease, in particular in IPF, is less well understood. Although excessive and dysregulated procoagulant and antifibrinolytic activity has been documented in patients with IPF (Martinez et al, 2005; Gunther et al, 2000; Perez et al, 1993) accumulation of fibrin, often visible as hyaline membranes in ALI, is less well documented in these patients (verbal communication by Dr A.G. Nicholson, NHLI, London). Fibrin deposition requires the

presence of vascular-derived thrombin and fibrinogen implying that vascular leak is not an ongoing feature in stable IPF. Hyaline membranes are however a well-recognised feature of ALI/ARDS which is characterised by initial widespread lung injury resulting in diffuse alveolar damage with leakage of protein rich fluids from capillaries into the alveolar space (reviewed in (Ware et al, 2006)). Whilst IPF is characterised by a stable decline in respiratory failure some patients with IPF undergo acute deteriorations in respiratory function, known as acute exacerbations which are pathologically and clinically very reminiscent of ARDS (Collard et al, 2007). It has therefore been recently proposed that vascular leak and fibrin deposition may be major features of acute exacerbations in IPF patients contributing to the worsening in pathology (Collard et al, 2007). Locally produced FX/FXa may therefore contribute to the increase in procoagulant activity in IPF. Once activated FXa, or indeed, the TF/FVIIa/FXa complex is then capable of activating PAR<sub>1</sub> or PAR<sub>2</sub>, leading to a number of downstream cellular effects which will be discussed in the subsequent section.

## **4.2. The role of FXa in fibroblast to myofibroblast differentiation**

### **4.2.1. FXa induces the differentiation of lung fibroblasts to myofibroblasts**

There is compelling evidence that the primary effector cell in fibrosis is the myofibroblast—a contractile and highly synthetic, spindle-shaped cell, characterised by the *de novo* expression of  $\alpha$ -SMA positive stress fibres. Myofibroblasts are a major source of excessive production of collagen and other ECM proteins. Several studies have demonstrated that the myofibroblast population is increased in the lungs of patients with pulmonary fibrosis and that the number of myofibroblasts correlates with disease severity (Zhang et al, 1996; Kapanci et al, 1995). Identifying mediators and interfering with pathways that lead to the expansion of the myofibroblast population within the IPF lung has thus been a major focus of research within this area (reviewed in (Scotton C.J. and Chambers, 2007)).

The work presented in this thesis examined the role of FXa in myofibroblast differentiation *in vitro* by assessing the effect of FXa on  $\alpha$ -SMA mRNA and protein levels. Using primary fibroblasts derived from human lung explants, this study reports for the first time that activation of lung fibroblasts by FXa promotes their differentiation into  $\alpha$ -SMA positive myofibroblasts. Furthermore, this study provides compelling evidence that FXa induces myofibroblast differentiation via the activation of PAR<sub>1</sub> and the upregulation of the matricellular protein TSP-1 and subsequent activation TGF- $\beta$ . Although FXa can signal via PAR<sub>1</sub> or PAR<sub>2</sub> (or both) depending on cell type and co-



factor expression (Blanc-Brude et al, 2005; Riewald and Ruf, 2001), the data presented here adds further evidence that in terms of fibroblast function, PAR<sub>1</sub> plays a dominant role. Importantly, PAR<sub>1</sub>-deficient mice are significantly protected from bleomycin-induced fibrosis (Howell et al, 2005). Although previous work in the host laboratory suggested that thrombin might be the major physiological activator of PAR<sub>1</sub> in the context of lung fibrosis (Howell et al, 2002), the data presented here raise the likelihood that locally-produced and activated FX is equally a credible physiological activator of PAR<sub>1</sub> in this disease setting.

The effects of FXa on myofibroblast differentiation were found to be both concentration- and time-dependent and independent of changes in fibroblast cell number. The stimulatory effects of FXa were first observed at concentrations of 10nM with maximal stimulatory effects obtained between 25 and 50nM. These concentrations are similar to the range of concentrations of FXa previously described to mediate lung fibroblast proliferation and procollagen production (Blanc-Brude et al, 2005) as well as mediating the mitogenic effect of FXa on smooth muscle cells and endothelial cells (Herbert et al, 1998; Nicholson et al, 1996). Upregulation of  $\alpha$ -SMA by FXa was not observed until 36 hours at the protein level which was preceded by an increase in  $\alpha$ -SMA mRNA levels at 24 hours. These findings are consistent with similar kinetics previously observed for thrombin in mediating the differentiation of lung fibroblasts to myofibroblasts which was observed from 24 hours onwards (Bogatkevich et al, 2001). Furthermore, the magnitude of the response obtained with FXa stimulation was very similar to that obtained for thrombin at equimolar concentrations of both proteinases (work presented here), suggesting that FXa is as efficient at stimulating myofibroblast differentiation as thrombin.

During the course of the work performed for this thesis, a report was published suggesting that stimulation of lung fibroblasts with high doses of thrombin up to 100 nM induces  $\alpha$ -SMA expression in lung fibroblasts within 2 hours of stimulation (Zhang et al, 2005). The mechanism of induction appears to be different to that reported in this thesis and by (Bogatkevich et al, 2001). The authors propose that high concentrations of thrombin regulate  $\alpha$ -SMA expression at a posttranscriptional-rather than at a transcriptional level via the cold shock domain protein YB-1, a known repressor of  $\alpha$ -SMA transcription but which also binds mRNA and thus regulates the translational efficiency of  $\alpha$ -SMA (Zhang et al, 2005). Although the authors did not analyse the signalling receptor involved, high levels of thrombin such as 100 nM are known to activate other PARs (e.g. PAR<sub>4</sub>) and also act via non receptor mechanisms (reviewed

in (Coughlin, 1999)). The mechanism proposed in this thesis and by (Bogatkevich et al, 2001) suggest that transcriptional regulation of  $\alpha$ -SMA is mediated via the activation of PAR<sub>1</sub> which would explain the difference in observed mechanisms. It is also worth mentioning that this is a very high concentration of thrombin and whether such concentrations can be generated in extravascular compartments remains an unresolved issue.

The stimulatory effects of FXa on myofibroblast differentiation in primary adult human lung fibroblasts was confirmed in other fibroblast lines, including human fetal and mouse lung fibroblasts, indicating that the effects of FXa on myofibroblast differentiation are not restricted to primary adult human lung fibroblasts (pHALFs). In all experiments performed in this thesis, FXa consistently stimulated  $\alpha$ -SMA expression, however, the basal expression rates of pHALFs and their responsiveness to this proteinase varied between experiments with responses ranging from as high as 40-fold to as low as 3-fold at an optimised concentration of FXa of 10nM. This difference in responsiveness did not seem to be related to differences in cell number but rather appeared to be dependent on the different stages of passage of cells with older cells gradually losing their responsiveness. Although great care was taken to keep cell culture conditions as constant as possible, variability in cell responsiveness is a problem in particular when working with primary cells as these are not immortalised and undergo rapid cell senescence in culture with increasing passage number.

Apart from their pivotal role in ECM deposition, myofibroblasts also acquire a contractile phenotype. This is particularly apparent during the process of wound healing, where myofibroblasts are responsible for the force generation resulting in wound contraction and connective tissue retraction (reviewed in (Desmouliere et al, 2005), also see Introduction **Section 1.5.2**). Contraction of myofibroblasts and remodelling of connective tissue is thought to play a role in pulmonary fibrosis. Evidence for this is reflected in the pathological stiffness of lungs of patients with pulmonary fibrosis (reviewed in (King, Jr. et al, 2001)). Although the ability of the myofibroblasts to contract following FXa stimulation was not directly assessed during the course of this thesis, immunocytofluorescence studies were used to visualise the expression and assembly of  $\alpha$ -SMA fibres, since these are directly linked to an increase in the contractile phenotype of the myofibroblast (Arora and McCulloch, 1994; Hinz et al, 2001). Untreated fibroblasts revealed a low level of expression of  $\alpha$ -SMA that did not appear to be polymerised. In contrast,  $\alpha$ -SMA expression in cells stimulated by FXa assembled into an extensive network of stress fibres characteristic

of the myofibroblast phenotype, supporting the notion that FXa induces the differentiation of fibroblast into contractile  $\alpha$ -SMA positive myofibroblasts.

#### **4.2.2. FXa exerts its stimulatory effects on myofibroblast differentiation via PAR<sub>1</sub> coupling to G $\alpha_q$**

FXa and other coagulation proteinases, such as thrombin, exert their cellular effects via the activation of PARs by limited proteolytic cleavage of the N-terminal extracellular domain thereby unmasking a tethered ligand (Camerer et al, 2002) which by binding to the second extracellular loop initiates downstream signalling events. FXa is known to activate PAR<sub>1</sub> or PAR<sub>2</sub>, or both dependent on cell type and cofactor expression (reviewed in (Ruf et al, 2003)).

Experiments performed in this thesis to determine the mechanism by which FXa exerts its effects on  $\alpha$ -SMA expression examined the receptor mechanism involved in some detail. First, experiments were performed to determine the effect of the specific thrombin inhibitor, hirudin, on FXa-induced myofibroblast differentiation. Hirudin only had a small, but significant inhibitory effect on  $\alpha$ -SMA protein levels induced by FXa, indicating that FXa-induced myofibroblast differentiation occurs largely independent of thrombin signalling. The small stimulatory effect that appears to be thrombin mediated may be explained by activation of prothrombin by FXa due to prothrombin remaining either on the cell surface from the culture medium/serum or due to contamination of the FXa preparation. However, over 90% of FXa stimulatory activity could be blocked with the FXa inhibitor, antistasin suggesting that FXa exerts these effects via its proteolytic activity. I next examined the requirement for FXa catalytic activity using ASN, a specific natural inhibitor of FXa catalytic site isolated from leech *Haementeria offinalis* (Ohta et al, 1994). Short synthetic peptides mimicking the core inhibitory region of the molecule, including ASN D-Arg<sup>32</sup>-Pro<sup>38</sup>- (ASN core peptide), have been shown to be active and abolish the catalytic activity of FXa selectively (Ohta et al, 1994). These bind to the catalytic site of FXa in a reversible but highly specific fashion and compete with natural substrates. In addition, being derived from the naturally occurring molecule, ASN has the advantage of being active in complex physiological fluids. In the current study high molecular ratio of inhibitor/proteinase (16:1) was used to ensure efficient inhibition over the 36 hour incubation period of these myofibroblast differentiation experiments. These agonist/inhibitor ratios were based on previous work performed in our laboratory demonstrating that the effects of FXa on fibroblast proliferation and collagen production

were abrogated at these concentrations of ASN (Blanc-Brude et al, 2001;Blanc-Brude et al, 2005).

Experiments performed to characterize the PAR involved in FXa-mediated myofibroblast differentiation assessed the effect of the highly selective peptide agonists TFLLR and SLIGKV on  $\alpha$ -SMA expression. These agonists mimic the tethered ligand sequences of PAR<sub>1</sub> and PAR<sub>2</sub> respectively and therefore allow selective activation of the receptor, independent of receptor cleavage. TFLLR selectively activates PAR<sub>1</sub> and unlike the commonly used peptide SFLLR, it does not cross-activate PAR<sub>2</sub> in mesenchymal cells (Hollenberg et al, 1997). This is of particular importance given that the primary adult human lung fibroblasts used in this study were found to express PAR<sub>2</sub>. The work presented here shows that the synthetic activating peptide of PAR<sub>1</sub>, TFLLRN was capable of mimicking the stimulatory effects of FXa whilst the PAR<sub>2</sub> activating peptide had no effect. The concentration of TFLLRN needed to stimulate  $\alpha$ -SMA expression was at least three orders of magnitude higher than for FXa, however the maximum stimulation obtained was almost 50% lower when compared to FXa. Similar differences in potency and efficacy have been observed between coagulation proteinases and activating peptides for several other PAR<sub>1</sub>-mediated cellular effects (Chambers et al, 2000). There are several explanations that have been proposed to explain these differences. First, it is possible that following cleavage of PAR<sub>1</sub> by FXa the tethered ligand may adopt a more favourable position to ensure efficient activation of the receptor compared to free-floating activating peptide. Second, receptor cleavage *per se* may be necessary to ensure efficient activation. Third, it is possible that in addition to receptor cleavage, interactions of FXa with the receptor itself may be required. Forth, multiple rounds of receptor activation by FXa may be needed and peptides may only be short acting due to degradation over time. However the lack of an increased response with additional spiking of agonist throughout the time-course excludes a role for protein degradation.

Further indirect evidence for the importance of PAR<sub>1</sub> was provided by studies showing that FXa significantly induced the expression of  $\alpha$ -SMA in mouse lung fibroblasts, as these cells have been shown in this study and by others to only express PAR<sub>1</sub> and not PAR<sub>2</sub> (Bachli et al, 2003). Finally, critical evidence confirming a central role for PAR<sub>1</sub> in mediating FXa stimulatory effects on myofibroblast differentiation was obtained from studies utilising the potent and selective antagonist of PAR<sub>1</sub>, RWJ-58259 (Damiano et al, 2003). This is a small indole-based compound which specifically inhibits the tethered ligand of PAR<sub>1</sub> binding to its second extracellular loop, and thus prevents

receptor activation. RWJ-58259 has been shown to block PAR<sub>1</sub>-mediated signalling events both *in vitro* and *in vivo* (Andrade-Gordon et al, 2001). This inhibitor completely abrogated FXa-induced myofibroblast differentiation at  $\mu$ M concentrations similar to the IC<sub>50</sub> previously described to block thrombin-induced platelet responses in *in vitro* culture systems (Andrade-Gordon et al, 2001; Damiano et al, 2003). Taken together, these studies provide compelling evidence that PAR<sub>1</sub> is the major signalling receptor involved in FXa-induced myofibroblast differentiation.

Previous studies in our laboratory have shown that in terms of fibrogenic effects, PAR<sub>1</sub> is the major cell signalling receptor for FXa mediating fibroblast G-protein signalling, proliferation and extracellular matrix production (Blanc-Brude et al, 1999; Blanc-Brude et al, 2005). In heterologous transfection systems, Riewald and Ruf reported that FXa activates PAR<sub>1</sub> and PAR<sub>2</sub> (Riewald et al, 2001). PAR<sub>1</sub> activation by FXa was greatly enhanced by cotransfection of tissue factor and the addition of FVIIa. This group also showed that FXa can stimulate gene expression in HeLa cells that express PAR<sub>1</sub> and not PAR<sub>2</sub>. Consistent with these studies, the data presented here adds further evidence that in terms of fibroblast function, PAR<sub>1</sub> plays a dominant role. In contrast, current evidence suggests that PAR<sub>2</sub> plays an important role in mediating the effects of FXa on cancer cell migration and endothelial cell function (Jiang et al, 2004; Feistritzer et al, 2005).

PAR<sub>1</sub> mediates its pluripotent cellular effects by coupling to multiple G protein subunits including G $\alpha_q$ , G $\alpha_i$ , and G $\alpha_{12/13}$  (see Introduction **Section 1.9.2**). Although many of the resultant biological consequences of PAR<sub>1</sub> activation in fibroblasts are known, less is known about which G proteins mediate these events. Using the specific G $\alpha_i$  inhibitor pertussis toxin and a novel PAR<sub>1</sub> selective G $\alpha_q$  protein signalling antagonist, the work presented in this thesis shows that G $\alpha_q$  but not G $\alpha_i$  was necessary for PAR<sub>1</sub>-mediated myofibroblast differentiation. Together with unpublished data from our laboratory examining PAR<sub>1</sub>-activation induced CCL2 release in lung fibroblasts (Deng et al, unpublished data) this finding suggests that G $\alpha_q$  plays an important role in mediating the stimulatory effects of PAR<sub>1</sub> on fibroblast function. Interestingly, a recent report has suggested that there may be important differences between thrombin and peptide agonists in terms of subsequent ability of PAR<sub>1</sub> to activate different G-proteins (McLaughlin et al, 2005b). This may potentially further explain the differences in  $\alpha$ -SMA expression observed between FXa and TLLR stimulation. Furthermore, previous studies by the host laboratory examining the effect of FXa on fibroblast proliferation suggested that this effect is mainly G $\alpha_i$  mediated (Blanc-Brude et al, 2005). It is

therefore tempting to speculate that FXa may induce different fibroblast responses by selectively recruiting different G proteins.

#### **4.2.3. FXa induces myofibroblast differentiation via a TGF- $\beta_1$ -dependent mechanism in vitro**

FXa elicits a number of its cellular responses, including pro-inflammatory and pro-fibrotic effects via the release of secondary mediators. For example FXa exerts its potent fibroblast mitogen effects via the autocrine release of PDGF (Blanc-Brude et al, 2001). Consistent with these observations, myofibroblast differentiation induced in response to FXa is a delayed response, raising the possibility that FXa exerts its effects via the autocrine production and/or activation of a secondary mediator (Cogan et al, 2002; Chambers et al, 2003; Subramanian et al, 2004). TGF- $\beta_1$  is one of the major cytokines that has been implicated in myofibroblast differentiation and experiments performed during the course of this thesis provided strong evidence that FXa mediated fibroblast to myofibroblast differentiation is dependent on TGF- $\beta_1$  activity.

The initial studies of this thesis assessed if FXa was capable of producing and/or activating TGF- $\beta_1$  in primary human lung fibroblasts. TGF- $\beta_1$  is a potent cytokine which is secreted in an inactive form (reviewed in (Sheppard, 2001a) and activation of TGF- $\beta_1$  is a major rate limiting step in the regulation of TGF- $\beta_1$  bioavailability (see Introduction **Section 1.6.2.**). Active TGF- $\beta_1$  was measured using a bioassay, based on mink lung epithelial cells stably transfected with truncated TGF- $\beta_1$ -responsive PAI-1 promoter fused to a luciferase reporter gene and capable of detecting concentrations of active TGF- $\beta_1$  as low as 0.2pM (Abe et al, 1994). In these experiments performed here, it was not possible to test the effect of FXa since FXa interfered with the bioassay; however activation of PAR<sub>1</sub> with TFLLR was shown to induce the activation of TGF- $\beta_1$  in primary human lung fibroblasts. Further evidence for a role of FXa in mediating activation of TGF- $\beta_1$  was provided by studies assessing the effect of FXa stimulation on TGF- $\beta_1$ -mediated signalling pathways in fibroblasts. TGF- $\beta_1$  induces cell signalling by binding to TGF $\beta$ RII which causes the recruitment and activation of TGF $\beta$ IR (ALK5) in fibroblasts and subsequently induces SMAD 2/3 phosphorylation (Franzen et al, 1993; ten et al, 1993). Phosphorylated SMAD 2/3 then associates with SMAD 4, and translocates to the nucleus where this complex modulates the transcription of a large number of genes (Miyazono et al, 2000). SMAD signalling is generally held to be the major pathway activated in response to TGF- $\beta$  and involved in mediating TGF- $\beta$ 's profibrotic effects (Kapoun et al, 2006). The studies performed here demonstrate that stimulation of

primary lung fibroblasts with FXa induced the phosphorylation of SMAD2/3 in a time-dependent manner and provide evidence that FXa mediates the activation of TGF- $\beta_1$  in cultured lung fibroblast. Furthermore, there was no apparent change in TGF- $\beta_1$  mRNA levels following FXa stimulation. Together these studies suggest that the activation of TGF- $\beta_1$  rather than production is the main mechanism by which FXa regulates the bioavailability of TGF- $\beta_1$  in human lung fibroblasts.

Several activation mechanisms for TGF- $\beta_1$  have been described including that mediated by the potent extracellular matrix protein TSP-1 (reviewed in (Murphy-Ullrich and Poczatek, 2000), also see Introduction **Section 1.6.3.**). TSP-1 is known to activate latent TGF- $\beta_1$  by binding to the LSKL region of the LAP thus inducing a conformational change in the LAP in such a way that LAP bound to TSP-1 is unable to confer latency to TGF- $\beta_1$  (Schultz-Cherry and Murphy-Ullrich, 1993). In the work presented in this thesis, FXa was found to upregulate TSP-1 both at the mRNA and protein levels. The earliest increase in TSP-1 expression preceded FXa-induced SMAD2/3 phosphorylation. Importantly, the blocking peptide LSKL, previously shown to prevent the activation of TGF- $\beta_1$  by TSP-1 *in vitro* and *in vivo* (Ribeiro et al, 1999; Kondou et al, 2003), completely blocked PAR<sub>1</sub>-mediated TGF- $\beta_1$  activation as measured by the Mink lung bioassay, indicating that TSP-1 is involved in mediating the activation of TGF- $\beta_1$  by FXa. Although, previous studies have shown that activation of PAR<sub>1</sub> by thrombin increases TSP-1 mRNA levels in endothelial cells (McLaughlin et al, 2005a), this is the first study to directly link the upregulation of TSP-1 to the subsequent activation of latent TGF- $\beta_1$ .

A direct role for TGF- $\beta_1$  in FXa-induced myofibroblast differentiation was assessed by using two pharmacologically unrelated selective small molecule inhibitors of ALK-5, SB431542 (Callahan et al, 2002) and SD-208 (Uhl et al, 2004). SB431542 inhibits ALK5 and also two other types of type I receptors of the TGF- $\beta$  superfamily, the activin type I receptor ALK4 and the nodal type I receptor ALK7 (Inman et al, 2002a). SB431542 has been shown to have selectivity for ALK receptor kinases *in vitro* (IC<sub>50</sub> ~1 $\mu$ M) and only weakly inhibits MAP kinase p38 $\alpha$  with an IC<sub>50</sub> of ~10 $\mu$ M (Inman et al, 2002a). However, previous studies have provided convincing evidence that p38 MAP kinase is not involved in TGF- $\beta_1$ -mediated  $\alpha$ -SMA expression in lung fibroblasts (Kapoun et al, 2006). SD-208 is a 2,4-disubstituted pteridine-derived, ATP-competitive inhibitor (IC<sub>50</sub> of ~35 nM) which is selective for TGF- $\beta$ IR kinase by >100-fold compared with TGF- $\beta$ RII kinase and at least 20-fold over a panel of related protein kinases such as c-Jun NH2-terminal kinase, extracellular signal-regulated kinase, MAP kinase p38,

and epidermal growth factor receptor kinase (Bonniaud et al, 2005). *In vivo*, oral administration of SD-208 has previously been shown to significantly inhibit the progression of fibrosis in a rat model of TGF- $\beta_1$ -mediated lung fibrosis (Bonniaud et al, 2005). In the present study, SB431542 and SD-208 completely blocked FXa-induced  $\alpha$ -SMA expression at micromolar and nanomolar ranges respectively and also blocked TGF- $\beta_1$ -mediated  $\alpha$ -SMA expression at similar concentrations. These concentrations were chosen based on previous studies in the literature examining the effect of SB431542 on TGF- $\beta_1$ -mediated fibroblast effects, including procollagen production and myofibroblast differentiation (Mori et al, 2004). Similar concentration were also used in studies blocking TGF- $\beta_1$ -induced PAI-1 protein levels with SD-208 in primary rat lung fibroblasts (Kapoun et al, 2006). In addition, both compounds inhibited  $\alpha$ -SMA expression by approximately 50% at concentrations close to the IC<sub>50</sub> previously reported for these compounds (Mori et al, 2004; Kapoun et al, 2006).

The proposed mechanism of FXa-mediated myofibroblast differentiation contrasts with that by which thrombin mediates myofibroblast differentiation. Bogatkevich and colleagues showed that thrombin mediated myofibroblast differentiation via the activation of PAR<sub>1</sub> but in a TGF- $\beta_1$ -independent manner (Bogatkevich et al, 2001). The interesting difference between this previous report and the data presented here with FXa may have several explanations. First, the present study employed a small molecule antagonist approach to block TGF- $\beta_1$ -induced signalling via ALK5 the efficiency of which may be greater compared to an antibody blocking strategy such as used in thrombin-induced myofibroblast differentiation studies. Further, TSP-1-mediated TGF- $\beta_1$  activation by FXa was only detectable when lung fibroblasts were grown in co-culture with transformed mink lung cells, indicating that close cell contact between the two cell types (the effector and the reporter cell line) is essential. This observation is consistent with the TSP-1 mechanism of activation of TGF- $\beta$  previously described which is thought to retain active TGF- $\beta_1$  bound to the extracellular matrix, thus restricting TGF- $\beta_1$  signalling close to the site of activation (see Introduction **Section 1.6.2.**). The high affinity of TGF- $\beta_1$  for its receptor together with the lack of release of active TGF- $\beta_1$  into the culture medium may explain why antibody blockade may not be an efficient strategy to interfere with TGF- $\beta_1$  signalling in these *in vitro* studies. Second, although FXa and thrombin can both signal via PAR<sub>1</sub> in fibroblasts, there is increasing evidence that these proteinases do not always induce identical cellular responses. For example, our laboratory has previously reported that thrombin and FXa mediate distinct intracellular calcium transients in fibroblasts (Blanc-Brude et al, 2005). Thrombin and activated protein C (APC) which both signal via PAR<sub>1</sub> in



perturbed endothelial cells have also been reported to mediate distinct biological effects (Riewald and Ruf, 2005). As previously mentioned, different agonists of PAR<sub>1</sub> have recently been shown to selectively activate different G-protein pathways by their ability to alter receptor/G protein binding (McLaughlin et al, 2005b). Taken together, these data support the concept that FXa-triggered signalling via PAR<sub>1</sub> may be distinct from thrombin signalling and this may explain why these proteinases mediate distinct cellular responses.

#### **4.2.4. *In vivo* evidence for FXa-induced myofibroblast differentiation via a TGF- $\beta$ and TSP-1-dependent mechanism**

Having provided convincing evidence that FXa mediates fibroblast to myofibroblast differentiation via the activation of PAR<sub>1</sub> and subsequent TSP-1-mediated activation of latent TGF- $\beta$ , further studies were performed to assess the potential relevance of this mechanism *in vivo*. To this end PAR<sub>1</sub>, TSP-1 and  $\alpha$ -SMA immunoreactivity was analysed in serial sections from human fibrotic and donor lung samples. The work presented in this thesis provides further evidence that the FXa-mediated mechanism of TGF- $\beta$ <sub>1</sub> activation via TSP-1 might be operative in human fibrotic lung disease as PAR<sub>1</sub>, TSP-1 and  $\alpha$ -SMA were found to be co-expressed by myofibroblasts within fibrotic foci in IPF lung sections. It is worth pointing out that this mechanism of TGF- $\beta$ <sub>1</sub> activation is likely to be highly specific to fibroblasts within these regions as there was no TSP-1 staining on hyperplastic epithelial cells within the same tissue samples. There is further good evidence that the  $\alpha$ v $\beta$ 6 integrin mechanism is the major activation mechanism involved in TGF- $\beta$ <sub>1</sub> activation by epithelial cells (Munger et al, 1999) so that the absence of TSP-1 in these regions is consistent with this alternative mode of TGF- $\beta$ <sub>1</sub> activation. During the course of this thesis, our laboratory working in collaboration with Dr. Jenkins showed that PAR<sub>1</sub> ligation on epithelial cells can lead to the activation of TGF- $\beta$ <sub>1</sub> via an  $\alpha$ v $\beta$ 6 integrin-dependent mechanism (Jenkins et al, 2000), so that PAR<sub>1</sub> activation on several cell types may play a pivotal role in driving the fibrotic response to lung injury by activating TGF- $\beta$ <sub>1</sub>.

### **4.3. Role of FXa in bleomycin-induced lung injury and fibrosis**

#### **4.3.1. ZK 807834**

In order to determine if FXa was causally involved in the development of pulmonary fibrosis *in vivo*, FXa inhibition studies were performed in the bleomycin model of lung injury and fibrosis. To this end a suitable pharmacological tool had to be identified. A number of direct FXa inhibitors have recently been developed as a novel strategy to

target specific components to the coagulation cascade to provide safer anticoagulants. There are two main strategies for inhibiting FXa that are currently being pursued clinically: direct inhibitors and indirect inhibitors which rely on antithrombin as a cofactor (Karnicki et al, 2004). ZK 807834 is a direct inhibitor of FXa and can inactivate both free FXa and FXa that is complexed with either TF/FVIIa, or FVa (the prothrombinase complex) (Light and Guilford, 2001). This is in contrast to indirect FXa inhibitors, such as fondaparinux or enoxaparin, which are unable to inactivate FXa which is part of a complex, as these indirect inhibitors act by bridging antithrombin to FXa which has no inhibitory effects on the complex (Bauer et al, 2002). ZK 807834 was therefore chosen to evaluate the effect of attenuating FXa activity in the bleomycin model of lung injury and fibrosis, since this compound will block FXa directly in the multiple conformations that may be particularly relevant in the extravascular space, such as the potent TF/FVIIa/FXa signalling complex (reviewed in (Riewald and Ruf, 2002)). Furthermore, ZK 807834 is highly selective for FXa ( $K_i = 0.11\text{nM}$ , compared with its  $K_i$  for thrombin of 2020 nM, (Light and Guilford, 2001)) and this selective nature was critical to ensure lack of inhibition of other proteinases such as thrombin which are known to influence the fibrotic response in this model (Howell et al, 2001). In addition, this compound has been shown to be effective *in vivo* and has anti-thrombotic activity in experimental models of venous and arterial thrombosis (Abendschein et al, 2000;Karnicki et al, 2004).

#### **4.3.2. ZK 807834 blocks the pro-fibrotic effects of FXa in vitro**

An important initial consideration was whether ZK 807834 would be capable of inhibiting FXa-induced cellular effects in the fibrotic lung and in particular fibroblast responses. Therefore the efficacy of ZK 807834 in blocking FXa-induced fibroblast proliferation and myofibroblast differentiation *in vitro* was initially assessed. In these experiments, ZK 807834 significantly inhibited FXa-induced fibroblast proliferation and myofibroblast differentiation in a concentration-dependent manner from equimolar concentrations of ZK 807834 onwards. The effects of ZK 807834 were comparable to those achieved with antistasin on myofibroblast differentiation in this thesis and to PPACK and rTAP on fibroblast proliferation used in previous studies in our laboratory (Blanc-Brude et al, 2005). In addition, at a concentration of 10nM, ZK 807834 inhibited FXa-induced  $\alpha$ -SMA protein levels by about 50%. This is consistent with a previous *in vitro* study reporting ZK 807834 inhibition of FXa in clot-bound prothrombinase with an average  $IC_{50}$  of 10 nM (Post et al, 2002).

#### **4.3.3. Effect of ZK 807834 on lung collagen accumulation in bleomycin-induced lung injury and fibrosis**

For *in vivo* studies, mice were given either bleomycin (1mg/kg) or saline by oropharyngeal administration followed by 50 mg/kg ZK 807834 or vehicle by intraperitoneal injection twice daily in the first week and then once a day in the second week until the end of the experiment. This dosing regime and route of administration were chosen based on the pharmacokinetic data provided by Berlex, indicating that a circulating concentration of ZK 807834 above 2 $\mu$ M was sufficient to double the prothrombin time in C57Bl/6J mice. In addition, real-time RT-PCR and immunohistochemistry studies presented in this thesis suggested that FX/FXa is generated chronically after-bleomycin injury; so that continuous FXa inhibition would be necessary. ZK 807834 was administered 24 hours prior to mice receiving a single oropharyngeal dose of saline or bleomycin so that a steady-state concentration of the drug would have been established at the time of oropharyngeal challenge. The experimental end point was 14 days, a time point at which extensive fibrotic lesions and an approximate doubling in total lung collagen can be observed (unpublished data in our laboratory).

These experiments predictably proved to be very difficult due to bleeding complications. Analysis of the animal's body weight, which is generally thought to be a good surrogate marker for the animal's welfare, revealed that ZK 807834 was delivered at a dose that was well tolerated. However, several animals in the saline/ZK 807834 and in the bleomycin/ZK 807834 group had to be sacrificed during the course of the experiment due to intraperitoneal haemorrhage thought to arise from the technical challenge of performing multiple intraperitoneal injections with an anticoagulant drug. This led to the decision to decrease the number of i.p injections to one per day only in the second week. This visibly reduced any haemorrhagic lesions close to the site of injection in the remaining animals. After 14 days, the effect of ZK 807834 on lung collagen accumulation in bleomycin-induced lung injury was evaluated. The data obtained showed that lung collagen accumulation at 14 days in mice treated with this compound was significantly attenuated. These observations provide strong-proof-of concept that FXa plays a significant role in the development of fibrosis in this model. During the course of these PhD studies, a study was published reporting that intratracheal gene delivery of TFPI, an endogenous inhibitor of TF/FVII and the ternary TF/FVIIa/FXa complex (Broze, Jr., 1995), also significantly attenuated lung collagen accumulation in the bleomycin model of lung injury and fibrosis in rats (Kijiyama et al,

2006). However, the present study is the first to show a direct functional link between FXa activity and the development of pulmonary fibrosis.

The data presented here contrasts with a previous study which assessed the effect of the FXa inhibitor enoxaparin in the bleomycin model of lung injury and fibrosis and showed no effect on collagen accumulation (Laxer et al, 1999). The differences in the effect of enoxaparin and ZK 807834 on fibrogenesis may have several explanations. First, and probably most important, similarly to fondaparinux, enoxaparin blocks FXa indirectly by influencing the activity of antithrombin III. This raises questions as to whether FXa was not efficiently targeted in the enoxaparin study as antithrombin III has no inhibitory actions against complexed FXa (i.e. TF/FVIIa/FXa). When assembled into the ternary complex, TF/FVII/FXa, FXa is 5-times more efficient at activating PAR<sub>1</sub> compared with free FXa (Riewald and Ruf, 2001). If FXa is predominantly present in the complexed form in the extravascular space, enoxaparin may not have effectively interfered with extravascular FXa activity. Second, the enoxaparin study evaluated collagen accumulation in mouse lungs by analysis of BALF and by histological, semi-quantitative image analysis of alveolar wall fraction and fibrosis fraction. The data presented here analysed total lung hydroxyproline content by the highly sensitive and quantitative HPLC method (Campa et al, 1990; Chambers et al, 1994). It is therefore possible that smaller differences in lung collagen accumulation may have been missed in the enoxaparin study.

#### ***4.3.4. Possible mechanisms by which FXa inhibition by ZK 807834 may have afforded protection in the bleomycin model of lung injury and fibrosis***

There are several mechanisms by which FXa inhibition may afford protection in the bleomycin model of lung injury and fibrosis. First, it is tempting to speculate that the stimulatory effects of FXa on myofibroblast differentiation demonstrated in this thesis may be a major mechanism by which FXa drives the fibrotic response to bleomycin-induced lung injury. Immunohistochemical studies presented in this thesis showing colocalisation of PAR<sub>1</sub>,  $\alpha$ -SMA and TSP-1 further lend some support to this notion in patients with IPF. Whilst this is an important question arising from the work in this thesis, due to time limitations there is currently no direct evidence to support this hypothesis. Second, the possibility that protection may have been partly influenced by an effect on inflammation cannot be ruled out at present. Inflammation plays an important role in driving the fibrotic response to lung injury and several studies have shown that blocking inflammation in the bleomycin model affords protection from subsequent lung collagen accumulation (Chen et al, 2006; Dik et al, 2003a).

Importantly, previous work in our laboratory has shown that PAR<sub>1</sub>-deficiency is associated with an attenuated inflammatory response as measured by the reduction in pro-inflammatory cytokines and protein leak (Howell et al, 2005). In addition, FXa is a potent inducer of pro-inflammatory cytokines such as IL-1 in fibroblasts (Jones and Geczy, 1990), IL-2 by lymphocytes (Altieri and Starnes, 1994), IL-6 and IL-8 in endothelial cells (Senden et al, 1998) and CCL2, IL-1 IL-8 and IL-6 in dermal fibroblasts (Bachli et al, 2003). Third, because of the central role of FXa in the coagulation cascade, direct FXa inhibition may also afford protection in this model by interfering with thrombin generation and thrombin-dependent procoagulant, as well as PAR<sub>1</sub>-mediated pro-fibrotic signalling. Several studies to date have shown that a reduction in thrombin generation leads to a substantial attenuation in the fibrotic response to lung injury (Howell et al, 2001; Yasui et al, 2001; Kijiyama et al, 2006). However, this study in combination with previous studies in our laboratory have clearly shown that FXa exerts its stimulatory effects on fibroblast to myofibroblast differentiation, proliferation and procollagen production independently of thrombin generation (Blanc-Brude et al, 2005). Thrombin levels in BALF have previously been reported to peak 6 days post-bleomycin injury (Howell et al, 2001). The work in this thesis shows for the first time that FX is locally expressed in the injured lung, and that its local production persists throughout the fibrotic phase of the bleomycin model of lung injury and fibrosis. This finding suggests that during the fibrotic phase of this model, FXa signalling may occur independently of thrombin generation, but further work is clearly needed to substantiate this claim. Fourth, a role for FXa procoagulant effects in this model cannot be excluded at present. Intra-alveolar accumulation of fibrin has been extensively documented in bleomycin-induced lung injury and fibrosis and in human studies of fibrotic lung disease (Idell et al, 1987; Chapman et al, 1986; Ikeda et al, 1989; Kotani et al, 1995). Fibrin in combination with fibronectin is thought to influence the tissue repair/fibrotic response by providing a provisional matrix, on which fibroblasts can proliferate and produce collagen (Pohl et al, 1979). This matrix contributes to alveolar collapse and traction of remaining airspaces (honeycombing). Furthermore, it binds fibrogenic factors and cytokines which are released during fibrinolysis (Grainger et al, 1995). However, the question of whether fibrin deposition plays a role in the development of fibrosis remains at present controversial as two independent studies have shown that mice deficient in fibrinogen are not protected from bleomycin-induced lung injury and fibrosis (Ploplis et al, 2000) (Hattori et al, 2000). These studies have led to the suggestion that fibrin *per se* may not be required for the progression of fibrosis in this model.

#### **4.3.5. Problems encountered during the assessment of ZK 807834 in the bleomycin model of lung injury and fibrosis**

In order to specifically block FXa activity in the extravascular space an attempt was made to administer ZK 807834 by oropharyngeal instillation. These experiments however were very unsuccessful with the majority of animals not surviving to the end of the study. Administration of the compound at a dose which attenuated collagen deposition when administered via multiple i.p. injections was associated with severe bleeding complications resulting in massive pulmonary haemorrhage and eventual death of animals. Interestingly, when the anticoagulant dose was reduced by five-fold, all mice survived the treatment and no apparent bleeding was observed on macroscopic inspection of the lungs. However, there was no significant effect on lung collagen accumulation in response to bleomycin indicating that this dosing regime may not have successfully blocked FXa signalling.

It is possible that the therapeutic window for FXa blockade is much narrower when targeted locally within the pulmonary compartment. In addition, although pharmacokinetic data was made available to us by Berlex for the administration of this compound via a parental route, this data was not available for oropharyngeal administration. It is therefore possible that the compound was not cleared efficiently within the pulmonary compartment and therefore accumulated. Furthermore, due to the low solubility of this compound, to achieve adequate concentrations of the inhibitor, it had to be administered in large volumes of saline (50µl) on a daily basis. This was poorly tolerated by the animals, especially in the first week of administration when lung oedema is highest. In summary, oropharyngeal administration of ZK 807834 was associated with massive bleeding complications and future studies are needed to evaluate the pharmacokinetic properties of this compound for this route of administration.

#### **4.4. Conclusions and implications**

This thesis examined the hypothesis that FX/FXa is locally upregulated in the fibrotic lung and contributes to the development of pulmonary fibrosis by influencing fibroblast function via the activation of PAR<sub>1</sub>. The work presented in this thesis reports the novel finding that FXa, a central proteinase of the coagulation cascade, is locally expressed and upregulated in experimentally-induced lung injury and in IPF. Furthermore this thesis reports that FXa is a potent mediator of the myofibroblast differentiation programme via the activation of PAR<sub>1</sub> and subsequent TSP-1-mediated activation of

latent TGF $\beta$ . Finally, FXa blockade modulated the fibrotic response to experimentally-induced lung injury and fibrosis. These data provide a paradigm shift in our understanding of the origin of the procoagulant activity by providing an alternative mechanism for the generation of FXa via the local extravascular expression of the zymogen in the injured lung and further identifies FXa as a novel fibrogenic factor in the pathogenesis of pulmonary fibrosis (**Figure 4.1**).

#### **4.4.1. A novel origin for procoagulant activity in the lung**

The findings presented in this thesis provide broad-reaching implications to our current understanding of the generation of procoagulant activity in fibrotic lung disease. Importantly, they challenge the long-held perception of the origin of increased procoagulant activity in fibrotic lung disease, and shift the focus from the circulation as a major source of this activity to coagulation factors that are locally produced within the injured lung. This thesis focused on FX, however there is increasing evidence that other coagulation proteinases may be locally produced in the lung. Preliminary data in our laboratory suggest that FVII is locally produced in experimentally-induced lung injury and fibrosis (verbal communication by Dr Scotton) and a recent report indicates that FXII may be synthesized by alveolar epithelial cells in the lungs of patients with fibrotic lung disease (Wygrecka et al, 2007a). This raises the tantalizing possibility that the lung is capable of inducing an extravascular coagulation system in response to injury.

The lung has developed a complex set of strategies to limit injury and promote repair whereby the coagulation system in concert with the inflammatory system play major roles. Both systems are inextricably linked and are thus not only activated simultaneously but control each other. For example excessive activation of the coagulation cascade often follows systemic inflammatory states and some components of the coagulation cascade are always activated in patients with septic shock (reviewed in (Riewald and Ruf, 2002)). It is thus tempting to speculate that a local inducible coagulation system has evolved in parallel with the inflammatory system to work in concert providing homeostatic balance in the extravascular space.

#### **4.4.2. FXa is a novel pathogenic factor in fibrotic lung disease**

Much of the work in the field of coagulation thus far has provided compelling evidence for a central role for thrombin in the development of pulmonary fibrosis (Howell et al, 2001). A role for coagulation proteinases upstream of thrombin such as FXa has long been suspected, however this is the first study to suggest a direct functional link

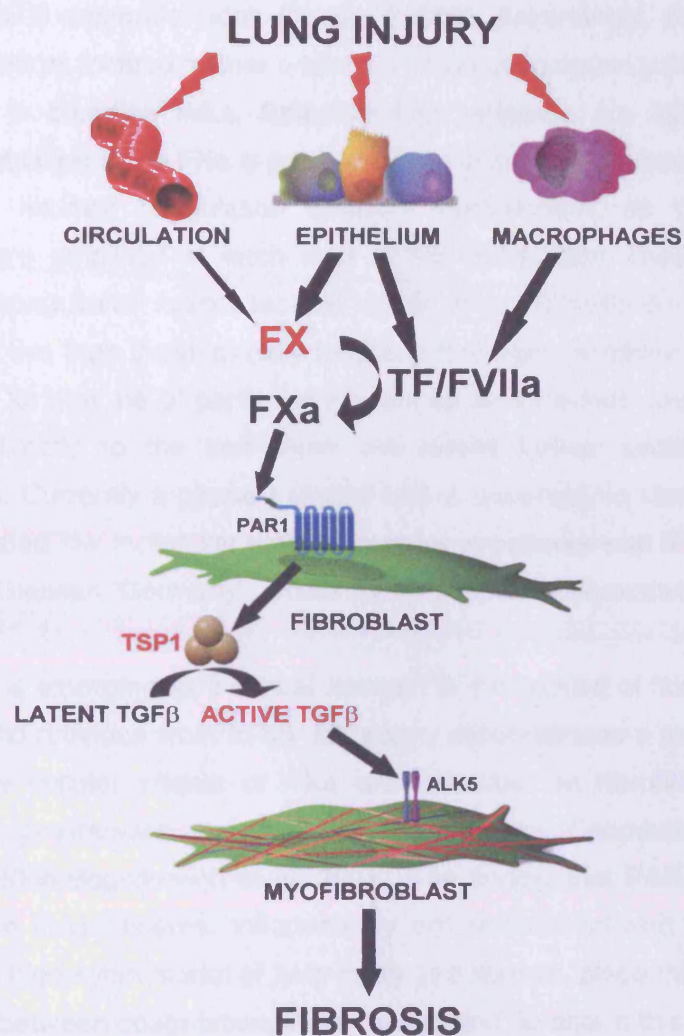
between FXa and the development of pulmonary fibrosis. The work presented in this study together with previous work in our laboratory provides further compelling evidence that PAR<sub>1</sub> is the major signalling receptor by which this proteinase exerts its pro-fibrotic effects on fibroblasts. Further studies are needed to confirm the relative *in vivo* importance of PAR<sub>1</sub> signalling by FXa, however recent reports in our laboratory have shown that PAR<sub>1</sub>-deficient mice are significantly protected from bleomycin-induced fibrosis (Howell et al, 2005). It has previously been proposed that thrombin might be the major physiological activator of PAR<sub>1</sub> in this model based on the evidence that direct thrombin inhibition is also protective (Howell et al, 2001), however the data presented here raise the possibility that circulation-derived/and or locally-produced FX/FXa in concert with TF and FVII could generate sufficient extravascular FXa to be a credible physiological activator of this receptor in the context of fibrotic lung disease.

#### **4.4.3. Possible therapeutic implication for antifibrotic compounds**

There is overwhelming evidence that the coagulation cascade plays a central role in driving the pro-inflammatory and pro-fibrotic effects in response to experimental lung injury. Some evidence that targeting the coagulation cascade in human fibrotic lung disease may be beneficial has recently been provided by a small non-blinded, randomized trial of 56 patients with IPF given prednisolone alone or prednisolone plus anticoagulation (oral warfarin for outpatients or low molecular weight heparin for hospitalized patients)(Kubo et al, 2005). The investigators report a significant increase in survival in the anticoagulant group, with 63% survival at 3 years in the anticoagulant group versus 35% in the non-anticoagulant group. Both patient groups had a similar incidence of acute exacerbations, but mortality associated with acute exacerbation was lower in the anticoagulant group (18% versus 71%). Despite some methodological caveats (e.g. possible selection bias towards advanced disease, concerns regarding patient randomization etc.), this remains one of the most significant beneficial outcomes on survival in an IPF clinical study suggesting that disordered coagulation may be an important component of acute exacerbations in IPF(Collard et al, 2007).

The work presented in this thesis has provides proof-of-principle that targeting FXa may be therapeutically beneficial in fibrotic lung disease. From a clinical perspective it is now increasingly recognised that therapeutic approaches based on selective inhibitors of specific coagulation factors, such as FXa rather than traditional, multi-targeted anticoagulants, such as warfarin and unfractionated heparin, are likely associated with a wider therapeutic window and therefore more effective, safer and easier to use (Bauer, 2006a;Bauer, 2006b).





**Figure 4.1. Proposed mechanism underlying the contribution of FX to lung fibrosis**

Following lung injury, both locally produced as well as circulation-derived FX contributes to the increase in FXa activity in the fibrotic lung. FXa induces its profibrotic effects via the activation of PAR<sub>1</sub> and subsequent differentiation of fibroblasts into the myofibroblast phenotype. This is dependent on the transcriptional induction of TSP-1 leading to the activation of the major profibrotic cytokine TGF-β<sub>1</sub> in a TSP-1-dependent manner.

The recombinant hirudins, which target thrombin, were the first anticoagulants to be developed that target a specific coagulation factor. However, direct thrombin inhibitors have a narrow therapeutic index (Bauer, 2006b). Accordingly, selective inhibition of coagulation factors located further upstream in the coagulation pathway might be safer with respect to bleeding risks. Selective FXa inhibitors are felt to be effective at blocking coagulation since FXa is positioned at the start of the common pathway of the extrinsic and intrinsic coagulation systems. Furthermore, as the levels of serine proteinases are amplified at each step of the coagulation cascade, anticoagulants which target coagulation factors located higher up in the cascade, such as FXa, might be more effective than those directly targeting thrombin. In addition, alveolar epithelial cell-derived FXa may be of particular interest as an inhalation route would deliver the drug more directly to the epithelium and would further avoid potential bleeding complications. Currently a phase I clinical trial is underway in Germany to assess the effects of inhaled low molecular weight heparins in patients with IPF (Andreas Günther, University of Giessen, Germany), which will no doubt be informative.

Finally, PAR<sub>1</sub> is emerging as a critical receptor in the context of fibrotic lung conditions. This thesis and previous work in our laboratory demonstrates a major role for PAR<sub>1</sub> in mediating the cellular effects of FXa and thrombin on fibroblast to myofibroblast differentiation, proliferation and procollagen production (Chambers et al, 1998; Blanc-Brude et al, 2005; Bogatkevich et al, 2001). The finding that PAR<sub>1</sub> knockout mice are protected from lung oedema, inflammatory cell recruitment and the development of fibrosis in the bleomycin model of lung injury and fibrosis, place this receptor central to the crosstalk between coagulation, inflammation and fibrosis in this model (Howell et al, 2005). Evidence that PAR<sub>1</sub> may play a major role in the context of human fibrotic lung disease was provided by observations in our and other laboratories showing that this receptor is highly upregulated on fibroblasts and macrophages within fibrotic foci in the lungs of patients with IPF (Howell et al, 2001) and pulmonary fibrosis associated with systemic sclerosis (Bogatkevich et al, 2005).

Work performed in collaboration with our laboratory also demonstrated a link between PAR<sub>1</sub> ligation on epithelial cells and the subsequent activation of the major profibrotic cytokine TGF- $\beta$ <sub>1</sub> via the  $\alpha$ v $\beta$ 6 integrin (Jenkins et al, 2006) and data presented here show that in fibroblasts, PAR<sub>1</sub> activation by FXa activates latent TGF- $\beta$ <sub>1</sub> via the transcriptional upregulation of TSP-1. The important role of PAR<sub>1</sub> in mediating TGF- $\beta$ <sub>1</sub> activation is of particular interest in terms of current drug targeting strategies in IPF. Given the important role of TGF- $\beta$ <sub>1</sub> in regulating inflammation and acting as a tumour

suppressor in certain contexts (see Introduction **Section 1.6.1.**), therapies aimed directly at TGF- $\beta_1$  as a drug target may be complicated by undesirable side-effects. Thus, targeting pathways involved in the activation of TGF- $\beta_1$  that are highly upregulated in fibrotic lung disease such as PAR $_1$ , may allow for successful blockade of TGF- $\beta_1$  profibrotic effects without significant impediment on its other biological functions involving different latent TGF- $\beta_1$  activation mechanisms.

The recent development of PAR $_1$  receptor antagonists could thus be of considerable interest in this area. PAR $_1$  blocking strategies are now increasingly credible therapeutic agents as in April 2006, Schering-Plough announced fast-track designation for a PAR $_1$  antagonist (SCH530348), after successful phase II clinical drug development, for assessment in its ability to reduce cardiovascular morbidity and mortality in patients with acute coronary syndromes (Schering-Plough Corporation 04/19/2006).

The work presented in this thesis further demonstrates the effectiveness of blocking FXa-induced fibroblast to myofibroblast differentiation using a recently developed novel small molecule inhibitor, which blocks PAR $_1$  at the intracellular interaction site with G $\alpha_q$  (Caden Biosciences) and therefore allows more selective targeting of some, but not all, PAR $_1$ -mediated cellular responses. Such an antagonist approach may therefore hold promise for selectively interfering with deleterious FXa signalling in the context of fibrotic lung disease, whilst preserving other essential PAR $_1$ -mediated cellular responses. In conclusion, the rationale for strategies aimed at blocking coagulation signalling is gaining strength and may provide new hope for the treatment of fibroproliferative lung disease.

#### 4.5. Future Studies

This thesis has provided compelling evidence that the coagulation proteinase FXa and its major fibroblast signalling receptor, PAR $_1$ , play important roles in the pathophysiology of pulmonary fibrosis. The discovery that the zymogen FX can be derived from a local tissue source rather than the circulation suggests that FXa may represent an important physiological activator of PAR $_1$  in extravascular compartments and may signal in the absence of thrombin generation and vascular leak. Further studies are needed to determine the molecular mechanisms that regulate locally produced FX in the fibrotic lung and the relative role it plays in the development of pulmonary fibrosis versus FXa derived from the circulation:

---

**a) The role of local FX in mediating the fibrotic response to bleomycin-induced lung injury**

To address the relative contribution of locally-produced FX in the pathogenesis of pulmonary fibrosis studies could be performed examining the effect of local knockdown of existing FX by short-hairpin RNA (shRNA) against FX in the bleomycin model of lung injury and fibrosis. Recent success in knocking down genes using shRNA in other models (Siner et al, 2007) suggest that this approach might be fruitful. Furthermore, the effect of bleomycin administration could be assessed by the generation of tissue specific knock-out mice, which do not express FX in alveolar epithelial cells.

**b) Mechanisms leading to increased expression of FX in the fibrotic lung**

Experiments performed in this thesis examining the potential mediators involved in regulating FX expression in fibrotic lung disease suggest that bleomycin increases FX mRNA levels in alveolar epithelial cells. Given that bleomycin exerts many of its pro-fibrotic effects via oxidative stress responses, further studies could be performed to examine the potential link between the generation of reactive oxygen species and the upregulation of FX mRNA levels. To this end alveolar epithelial cell lines and primary cells could be stimulated with a number of reagents, (e.g. hydrogen peroxide) that mimic oxidative stress environments and their effect on FX mRNA and protein levels could be measured. The antioxidant n-acetylcysteine has previously been shown to be beneficial in the bleomycin model of lung injury and fibrosis (Shahzeidi et al, 1991). To examine if oxidative stress mediates FX expression *in vivo* the effect of n-acetylcysteine on FX expression in this model could be assessed.

**c) The role of FXa in mediating EMT and fibrocyte recruitment**

The work presented in this thesis showed that FXa is a potent mediator of the myofibroblast differentiation programme. There is increasing evidence that a significant proportion of myofibroblasts can be produced from transdifferentiation of adjacent epithelial cells through a process known as epithelial-mesenchymal transdifferentiation (EMT) (see Introduction **Section 1.5.3.**). A role for thrombin in inducing EMT in alveolar epithelial cells was recently suggested (Ando et al, 2007) and plasmin promotes the transdifferentiation of murine tubular epithelial cells via activation of PAR<sub>1</sub> (Zhang et al, 2007). This has led to the hypothesis that FXa may play a role in mediating EMT. To address this hypothesis *in vitro*, experiments could assess the stimulatory effect of FXa on EMT in cultured alveolar epithelial cells.

**d) Differential downstream signalling effects of FXa and thrombin**

The results in this thesis suggest that the coagulation proteinases thrombin and FXa induce differential downstream signalling events. Whilst this study has provided compelling evidence for a role of TGF- $\beta_1$  in FXa-induced myofibroblast differentiation, previous reports by others suggest that thrombin mediates fibroblast function independently of TGF- $\beta_1$  activation (Bogatkevich et al, 2001). This is in agreement with previous studies performed in our laboratory demonstrating that although FXa, similarly to thrombin is also able to activate PAR<sub>1</sub> on lung fibroblasts, the calcium signals generated by thrombin-mediated PAR<sub>1</sub> activation are different compared to FXa-induced activation of the receptor (Blanc-Brude et al, 2005). To further understand the potentially different roles of these proteinases in influencing fibroblast function, transcriptional profiling studies comparing the stimulatory effects of thrombin and FXa on fibroblast could be examined.

**e) Mechanism of protection of FXa inhibition in bleomycin-induced lung injury and fibrosis**

The work presented in this thesis demonstrated that mice treated with the selective FXa inhibitor, ZK 807834, were significantly protected from lung collagen accumulation at 14 days. To begin to elucidate the mechanisms by which FXa inhibition affords protection in this model the following experiments could be performed. First, the effect of FXa inhibition on lung inflammation could be assessed. To this end, levels of serum and BALF pro-inflammatory cytokines, which are known to be induced following FXa stimulation such as IL-1, IL-6 and IL-8 (see Introduction **Section 1.8.3.**), could be assayed. Second, the effect of ZK 807834 on pulmonary vascular permeability could be further evaluated by the technique of Evans blue extravasation. Third, the effect of FXa inhibition on fibroblast to myofibroblast differentiation could be assessed by measuring  $\alpha$ -SMA mRNA and protein levels. Fourth, the relative contribution of each pathway to lung collagen accumulation could be assessed, by delivering ZK 807834 at different phases of the injury response following bleomycin instillation. Finally, to evaluate if FXa exerts its profibrotic effects via the activation of PAR<sub>1</sub> *in vivo*, the effect of FXa inhibition by ZK 807834 in PAR<sub>1</sub> null mice following bleomycin-induced lung injury could be assessed.

---

## Chapter 5: References

- Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB (1994). An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter- luciferase construct. *Anal Biochem* 216:276-284.
- Abe R, Donnelly SC, Peng T, Bucala R, Metz CN (2001). Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 166:7556-7562.
- Abendschein DR, Baum PK, Martin DJ, Vergona R, Post J, Rumennik G, Sullivan ME, Eisenberg PR, Light DR (2000). Effects of ZK-807834, a novel inhibitor of factor Xa, on arterial and venous thrombosis in rabbits. *J Cardiovasc Pharmacol* 35:796-805.
- Adamson IY, Bowden DH (1974). The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* 77:185-197.
- Adler KB, Low RB, Leslie KO, Mitchell J, Evans JN (1989). Contractile cells in normal and fibrotic lung. *Lab Invest* 60:473-485.
- Agostini C, Gurrieri C (2006). Chemokine/cytokine cocktail in idiopathic pulmonary fibrosis. *Proc Am Thorac Soc* 3:357-363.
- Ahnoff M, Grundevik I, Arfwidsson A, Fonselius J, Persson BA (1981). Derivatization with 4-chloro-7-nitrobenzofurazan for liquid chromatographic determination of hydroxyproline in collagen hydrolysate. *Anal Chem* 53:485-489.
- Allen JT, Knight RA, Bloor CA, Spiteri MA (1999). Enhanced insulin-like growth factor binding protein-related protein 2 (Connective tissue growth factor) expression in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. *Am J Respir Cell Mol Biol* 21:693-700.
- Altieri DC, Stamnes SJ (1994). Protease-dependent T cell activation: ligation of effector cell protease receptor-1 (EPR-1) stimulates lymphocyte proliferation. *Cell Immunol* 155:372-383.
- American Thoracic Society (2000). American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *Am J Respir Crit Care Med* 161:646-664.
- Ando S, Otani H, Yagi Y, Kawai K, Araki H, Fukuhara S, Inagaki C (2007). Proteinase-activated receptor 4 stimulation-induced epithelial-mesenchymal transition in alveolar epithelial cells. *Respir Res* 8:31.
- Antoniou KM, Pataka A, Bouros D, Siafakas NM (2007). Pathogenetic pathways and novel pharmacotherapeutic targets in idiopathic pulmonary fibrosis. *Pulm Pharmacol Ther* 20:453-461.
- Aragay AM, Collins LR, Post GR, Watson AJ, Feramisco JR, Brown JH, Simon MI (1995). G12 requirement for thrombin-stimulated gene expression and DNA synthesis in 1321N1 astrocytoma cells. *J Biol Chem* 270:20073-20077.
- Arora PD, McCulloch CA (1994). Dependence of collagen remodelling on alpha-smooth muscle actin expression by fibroblasts. *J Cell Physiol* 159:161-175.

- Asokanathan N, Graham PT, Fink J, Knight DA, Bakker AJ, McWilliam AS, Thompson PJ, Stewart GA (2002). Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. *J Immunol* 168:3577-3585.
- Babich M, King KL, Nissenson RA (1990). Thrombin stimulates inositol phosphate production and intracellular free calcium by a pertussis toxin-insensitive mechanism in osteosarcoma cells. *Endocrinology* 126:948-954.
- Bachhuber BG, Sarembock IJ, Gimple LW, Owens GK (1997). alpha-Thrombin induces transforming growth factor-beta1 mRNA and protein in cultured vascular smooth muscle cells via a proteolytically activated receptor. *J Vasc Res* 34:41-48.
- Bachli EB, Pech CM, Johnson KM, Johnson DJ, Tuddenham EG, McVey JH (2003). Factor Xa and thrombin, but not factor VIIa, elicit specific cellular responses in dermal fibroblasts. *J Thromb Haemost* 1:1935-1944.
- Bachofen M, Weibel ER (1982). Structural alterations of lung parenchyma in the adult respiratory distress syndrome. *Clin Chest Med* 3:35-56.
- Baffy G, Yang L, Raj S, Manning DR, Williamson JR (1994). G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. *J Biol Chem* 269:8483-8487.
- Bauer KA (2006a). New anticoagulants. *Hematology Am Soc Hematol Educ Program* 450-456.
- Bauer KA (2006b). New anticoagulants: anti IIa vs anti Xa--is one better? *J Thromb Thrombolysis* 21:67-72.
- Bauer KA, Hawkins DW, Peters PC, Petitou M, Herbert JM, van Boeckel CA, Meuleman DG (2002). Fondaparinux, a synthetic pentasaccharide: the first in a new class of antithrombotic agents - the selective factor Xa inhibitors. *Cardiovasc Drug Rev* 20:37-52.
- Baumgartner KB, Samet JM, Coultas DB, Stidley CA, Hunt WC, Colby TV, Waldron JA (2000). Occupational and environmental risk factors for idiopathic pulmonary fibrosis: a multicenter case-control study. Collaborating Centers. *Am J Epidemiol* 152:307-315.
- Baumgartner KB, Samet JM, Stidley CA, Colby TV, Waldron JA (1997). Cigarette smoking: a risk factor for idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 155:242-248.
- Behr J, Degenkolb B, Krombach F, Vogelmeier C (2002). Intracellular glutathione and bronchoalveolar cells in fibrosing alveolitis: effects of N-acetylcysteine. *Eur Respir J* 19:906-911.
- Benka ML, Lee M, Wang GR, Buckman S, Burlacu A, Cole L, DePina A, Dias P, Granger A, Grant B, . (1995). The thrombin receptor in human platelets is coupled to a GTP binding protein of the G alpha q family. *FEBS Lett* 363:49-52.
- Berridge MJ (1993). Inositol trisphosphate and calcium signalling. *Nature* 361:315-325.
- Bertozi P, Astedt B, Zenzius L, Lynch K, LeMaire F, Zapol W, Chapman HA, Jr. (1990). Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome [see comments]. *N Engl J Med* 322:890-897.
- Blanc-Brude OP, Archer F, Leoni P, Derian C, Bolsover S, Laurent GJ, Chambers RC (2005). Factor Xa stimulates fibroblast procollagen production, proliferation, and calcium signaling via PAR1 activation. *Exp Cell Res* 304:16-27.

- Blanc-Brude OP, Chambers RC, Archer F, Laurent GJ (1999). PAR-1 mediates factor Xa-induced cytosolic calcium mobilisation and mitogenesis in fibroblasts. p. A135.
- Blanc-Brude OP, Chambers RC, Leoni P, Dik WA, Laurent GJ (2001). Factor Xa is a fibroblast mitogen via binding to effector-cell protease receptor-1 and autocrine release of PDGF. *Am J Physiol Cell Physiol* 281:C681-C689.
- Bogatcheva NV, Garcia JG, Verin AD (2002). Molecular mechanisms of thrombin-induced endothelial cell permeability. *Biochemistry (Mosc)* 67:75-84.
- Bogatkevich GS, Tourkina E, Silver RM, Ludwicka-Bradley A (2001). Thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the proteolytically activated receptor-1 and a protein kinase C-dependent pathway. *J Biol Chem*.
- Bogatkevich GS, Gustilo E, Oates JC, Feghali-Bostwick C, Harley RA, Silver RM, Ludwicka-Bradley A (2005). Distinct PKC isoforms mediate cell survival and DNA synthesis in thrombin-induced myofibroblasts. *Am J Physiol Lung Cell Mol Physiol* 288:L190-L201.
- Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A (2005). PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* 120:303-313.
- Bonner JC (2004). Regulation of PDGF and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev* 15:255-273.
- Bonnaud P, Margetts PJ, Kolb M, Schroeder JA, Kapoun AM, Damm D, Murphy A, Chakravarty S, Dugar S, Higgins L, Protter AA, Gauldie J (2005). Progressive transforming growth factor beta1-induced lung fibrosis is blocked by an orally active ALK5 kinase inhibitor. *Am J Respir Crit Care Med* 171:889-898.
- Bonnaud P, Martin G, Margetts PJ, Ask K, Robertson J, Gauldie J, Kolb M (2004). Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. *Am J Respir Cell Mol Biol* 31:510-516.
- Bonnaud P, Margetts PJ, Kolb M, Haberberger T, Kelly M, Robertson J, Gauldie J (2003). Adenoviral Gene Transfer of Connective Tissue Growth Factor in the Lung Induces Transient Fibrosis. *Am J Respir Crit Care Med* 168:770-778.
- Border WA, Noble NA (1995). Targeting TGF-beta for treatment of disease. *Nat Med* 1:1000-1001.
- Bornstein P (1995). Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin 1. *J Cell Biol* 130:503-506.
- Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65:663-675.
- Brass LF, Pizarro S, Ahuja M, Belmonte E, Blanchard N, Stadel JM, Hoxie JA (1994). Changes in the structure and function of the human thrombin receptor during receptor activation, internalization, and recycling. *J Biol Chem* 269:2943-2952.
- Brocker V, Langer F, Fellous TG, Mengel M, Brittan M, Bredt M, Milde S, Welte T, Eder M, Haverich A, Alison MR, Kreipe H, Lehmann U (2006). Fibroblasts of recipient origin contribute to bronchiolitis obliterans in human lung transplants. *Am J Respir Crit Care Med* 173:1276-1282.



- Broekelmann TJ, Limper AH, Colby TV, McDonald JA (1991). Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci U S A* 88:6642-6646.
- Brouty-Boye D, Pottin-Clemenceau C, Doucet C, Jasmin C, Azzarone B (2000). Chemokines and CD40 expression in human fibroblasts. *Eur J Immunol* 30:914-919.
- Brown RD, Ambler SK, Mitchell MD, Long CS (2005). The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. *Annu Rev Pharmacol Toxicol* 45:657-687.
- Broze GJ, Jr. (1995). Tissue factor pathway inhibitor and the revised theory of coagulation. *Annu Rev Med* 46:103-112.
- Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A (1994). Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1:71-81.
- Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M (2001). Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* 22:199-204.
- Bunnett NW (2006). Protease-activated receptors: how proteases signal to cells to cause inflammation and pain. *Semin Thromb Hemost* 32 Suppl 1:39-48.
- Burns AR, Simon SI, Kukiela GL, Rowen JL, Lu H, Mendoza LH, Brown ES, Entman ML, Smith CW (1996). Chemotactic factors stimulate CD18-dependent canine neutrophil adherence and motility on lung fibroblasts. *J Immunol* 156:3389-3401.
- Butt RP, Laurent GJ, Bishop JE (1995). Collagen production and replication by cardiac fibroblasts is enhanced in response to diverse classes of growth factors. *Eur J Cell Biol* 68:330-335.
- Callahan JF, Burgess JL, Fornwald JA, Gaster LM, Harling JD, Harrington FP, Heer J, Kwon C, Lehr R, Mathur A, Olson BA, Weinstock J, Laping NJ (2002). Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5). *J Med Chem* 45:999-1001.
- Cambrey AD, Harrison NK, Dawes KE, Southcott AM, Black CM, du Bois RM, Laurent GJ, McAnulty RJ (1994). Increased levels of endothelin-1 in bronchoalveolar lavage fluid from patients with systemic sclerosis contribute to fibroblast mitogenic activity in vitro. *Am J Respir Cell Mol Biol* 11:439-445.
- Camerer E, Kataoka H, Kahn M, Lease K, Coughlin SR (2002). Genetic evidence that protease-activated receptors mediate factor Xa signaling in endothelial cells. *J Biol Chem* 277:16081-16087.
- Campa JS, McAnulty RJ, Laurent GJ (1990). Application of high-pressure liquid chromatography to studies of collagen production by isolated cells in culture. *Anal Biochem* 186:257-263.
- Campbell SE, Katwa LC (1997). Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. *J Mol Cell Cardiol* 29:1947-1958.
- Carver JR, Shapiro CL, Ng A, Jacobs L, Schwartz C, Virgo KS, Hagerty KL, Somerfield MR, Vaughn DJ (2007). American Society of Clinical Oncology clinical evidence review on the ongoing care of adult cancer survivors: cardiac and pulmonary late effects. *J Clin Oncol* 25:3991-4008.

- Chambers RC (2003). Role of coagulation cascade proteases in lung repair and fibrosis. *Eur Respir J Suppl* 44:33s-35s.
- Chambers RC, Dabbagh K, McAnulty RJ, Gray AJ, Blanc Brude OP, Laurent GJ (1998). Thrombin stimulates fibroblast procollagen production via proteolytic activation of protease-activated receptor 1. *Biochem J* 333:121-127.
- Chambers RC, Laurent GJ (2002). Coagulation cascade proteases and tissue fibrosis. *Biochem Soc Trans* 30:194-200.
- Chambers RC, Leoni P, Blanc-Brude OP, Wembridge DE, Laurent GJ (2000). Thrombin is a potent inducer of connective tissue growth factor production via proteolytic activation of protease-activated receptor-1. *J Biol Chem* 275:35584-35591.
- Chambers RC, Leoni P, Kaminski N, Laurent GJ, Heller RA (2003). Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *Am J Pathol* 162:533-546.
- Chambers RC, McAnulty RJ, Shock A, Campa JS, Newman Taylor AJ, Laurent GJ (1994). Cadmium selectively inhibits fibroblast procollagen production and proliferation. *Am J Physiol* 267:L300-L308.
- Chambers R, Laurent GJ (1997). Collagens. In: Crystal RG, Weibel ER, Barnes PJ, editors. *Philadelphia: Lippincott-Raven*, 1997. p. 709-727.
- Chamley JH, Campbell GR, McConnell JD, Groschel-Stewart U (1977). Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. *Cell Tissue Res* 177:503-522.
- Chan CP, Lin CP, Chang MC, Hsieh CC, Hsu CC, Lin CL, Jeng JH (1998). Effects of thrombin on the growth, protein synthesis, attachment, clustering and alkaline phosphatase activity of cultured human periodontal ligament fibroblasts. *Proc Natl Sci Counc Repub China B* 22:137-143.
- Chapman HA, Allen CL, Stone OL (1986). Abnormalities in pathways of alveolar fibrin turnover among patients with interstitial lung disease. *Am Rev Respir Dis* 133:437-443.
- Chapman HA, Jr., Allen CL, Stone OL, Fair DS (1985). Human alveolar macrophages synthesize factor VII in vitro. Possible role in interstitial lung disease. *J Clin Invest* 75:2030-2037.
- Chaponnier C, Gabbiani G (2004). Pathological situations characterized by altered actin isoform expression. *J Pathol* 204:386-395.
- Chen F, Gong L, Zhang L, Wang H, Qi X, Wu X, Xiao Y, Cai Y, Liu L, Li X, Ren J (2006). Short courses of low dose dexamethasone delay bleomycin-induced lung fibrosis in rats. *Eur J Pharmacol* 536:287-295.
- Chua F, Gauldie J, Laurent GJ (2005). Pulmonary fibrosis: searching for model answers. *Am J Respir Cell Mol Biol* 33:9-13.
- Cirino G, Cicala C, Bucci M, Sorrentino L, Ambrosini G, DeDominicis G, Altieri DC (1997). Factor Xa as an interface between coagulation and inflammation. Molecular mimicry of factor Xa association with effector cell protease receptor-1 induces acute inflammation in vivo. *J Clin Invest* 99:2446-2451.
- Cogan JG, Subramanian SV, Polikandriotis JA, Kelm RJ, Jr., Strauch AR (2002). Vascular smooth muscle alpha-actin gene transcription during myofibroblast

differentiation requires Sp1/3 protein binding proximal to the MCAT enhancer. *J Biol Chem* 277:36433-36442.

Coker RK, Laurent GJ, Shahzeidi S, Lympny PA, du Bois RM, Jeffery PK, McAnulty RJ (1997). Transforming growth factors-beta 1, -beta 2, and -beta 3 stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis. *Am J Pathol* 150:981-991.

Collard HR, Moore BB, Flaherty KR, Brown KK, Kaner RJ, King Jr TE, Lasky JA, Lloyd JE, Noth I, Oltman MA, Raghu G, Roman J, Ryu JH, Zisman DA, Hunnighake GW, Colby TV, Egan JJ, Hansell DM, Johkoh T, Martinez FJ (2007). Pulmonary Perspective: Acute Exacerbations of Idiopathic Pulmonary Fibrosis. *Am J Respir Crit Care Med*.

Colotta F, Sclaccia FL, Sironi M, Luini W, Rabiet MJ, Mantovani A (1994). Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. *Am J Pathol* 144:975-985.

Connolly AJ, Suh DY, Hunt TK, Coughlin SR (1997). Mice lacking the thrombin receptor, PAR1, have normal skin wound healing. *Am J Pathol* 151:1199-1204.

Cool CD, Groshong SD, Rai PR, Henson PM, Stewart JS, Brown KK (2006). Fibroblast Foci Are Not Discrete Sites of Lung Injury or Repair: The Fibroblast Reticulum. *Am J Respir Crit Care Med* 174:654-658.

Corrin B, Dewar A, Rodriguez-Roisin R, Turner-Warwick M (1985). Fine structural changes in cryptogenic fibrosing alveolitis and asbestosis. *J Pathol* 147:107-119.

Coughlin SR (2000). Thrombin signalling and protease-activated receptors. *Nature* 407:258-264.

Coughlin SR (1999). How the protease thrombin talks to cells. *Proc Natl Acad Sci U S A* 96:11023-11027.

Coughlin SR, Camerer E (2003). PARticipation in inflammation. *J Clin Invest* 111:25-27.

Coultas DB, Zumwalt RE, Black WC, Sobonya RE (1994). The epidemiology of interstitial lung diseases. *Am J Respir Crit Care Med* 150:967-972.

Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, Boivin GP, Bouck N (1998). Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* 93:1159-1170.

Crowston JG, Salmon M, Khaw PT, Akbar AN (1997). T-lymphocyte-fibroblast interactions. *Biochem Soc Trans* 25:529-531.

Damiano BP, Derian CK, Maryanoff BE, Zhang HC, Gordon PA (2003). RWJ-58259: a selective antagonist of protease activated receptor-1. *Cardiovasc Drug Rev* 21:313-326.

Darby I, Skalli O, Gabbiani G (1990). Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 63:21-29.

Dasch JR, Pace DR, Waegell W, Inenaga D, Ellingsworth L (1989). Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. *J Immunol* 142:1536-1541.

- Dawes KE, Cambrey AD, Campa JS, Bishop JE, McAnulty RJ, Peacock AJ, Laurent GJ (1996). Changes in collagen metabolism in response to endothelin-1: evidence for fibroblast heterogeneity. *Int J Biochem Cell Biol* 28:229-238.
- Dawes KE, Gray AJ, Laurent GJ (1993). Thrombin stimulates fibroblast chemotaxis and replication. *Eur J Cell Biol* 61:126-130.
- Dery O, Corvera CU, Steinhoff M, Bunnett NW (1998). Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol* 274:C1429-C1452.
- Derynck R, Zhang YE (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425:577-584.
- Desmouliere A, Chaponnier C, Gabbiani G (2005). Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 13:7-12.
- Desmouliere A, Darby IA, Gabbiani G (2003). Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab Invest* 83:1689-1707.
- Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122:103-111.
- Di Scipio RG, Hermodson MA, Davie EW (1977). Activation of human factor X (Stuart factor) by a protease from Russell's viper venom. *Biochemistry* 16:5253-5260.
- Dik WA, McAnulty RJ, Versnel MA, Naber BA, Zimmermann LJ, Laurent GJ, Mutsaers SE (2003a). Short course dexamethasone treatment following injury inhibits bleomycin induced fibrosis in rats. *Thorax* 58:765-771.
- Dik WA, Zimmermann LJ, Naber BA, Janssen DJ, Van Kaam AH, Versnel MA (2003b). Thrombin contributes to bronchoalveolar lavage fluid mitogenicity in lung disease of the premature infant. *Pediatr Pulmonol* 35:34-41.
- Dixit VM, Galvin NJ, O'Rourke KM, Frazier WA (1986). Monoclonal antibodies that recognize calcium-dependent structures of human thrombospondin. Characterization and mapping of their epitopes. *J Biol Chem* 261:1962-1968.
- Du J, Brink M, Peng T, Mottironi B, Delafontaine P (2001). Thrombin regulates insulin-like growth factor-1 receptor transcription in vascular smooth muscle: characterization of the signaling pathway. *Circ Res* 88:1044-1052.
- Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, Heath JK (1987). Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J* 6:1899-1904.
- Eickelberg O, Kohler E, Reichenberger F, Bertschin S, Woodtli T, Erne P, Perruchoud AP, Roth M (1999). Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *Am J Physiol* 276:L814-L824.
- Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH (1996). Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *Journal of Clinical Investigation* 97:232-237.

- Elias JA, Freundlich B, Adams S, Rosenbloom J (1990). Regulation of human lung fibroblast collagen production by recombinant interleukin-1, tumor necrosis factor, and interferon-gamma. *Ann N Y Acad Sci* 580:233-244.
- Feistritzer C, Lenta R, Riewald M (2005). Protease-activated receptors-1 and -2 can mediate endothelial barrier protection: role in factor Xa signaling. *J Thromb Haemost* 3:2798-2805.
- Feistritzer C, Riewald M (2005). Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood* 105:3178-3184.
- Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A (2006). IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* 12:99-106.
- Finigan JH, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, Ye SQ, Garcia JG (2005). Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. *J Biol Chem* 280:17286-17293.
- Fink L, Kinfe T, Seeger W, Ermert L, Kummer W, Bohle RM (2000a). Immunostaining for cell picking and real-time mRNA quantitation. *Am J Pathol* 157:1459-1466.
- Fink L, Kinfe T, Stein MM, Ermert L, Hanze J, Kummer W, Seeger W, Bohle RM (2000b). Immunostaining and laser-assisted cell picking for mRNA analysis. *Lab Invest* 80:327-333.
- Fink L, Seeger W, Ermert L, Hanze J, Stahl U, Grimminger F, Kummer W, Bohle RM (1998). Real-time quantitative RT-PCR after laser-assisted cell picking. *Nat Med* 4:1329-1333.
- Finlay GA, Thannickal VJ, Fanburg BL, Paulson KE (2000). Transforming growth factor-beta 1-induced activation of the ERK pathway/activator protein-1 in human lung fibroblasts requires the autocrine induction of basic fibroblast growth factor. *J Biol Chem* 275:27650-27656.
- Fiorucci S, Antonelli E, Distrutti E, Severino B, Fiorentina R, Baldoni M, Caliendo G, Santagada V, Morelli A, Cirino G (2004). PAR1 antagonism protects against experimental liver fibrosis. Role of proteinase receptors in stellate cell activation. *Hepatology* 39:365-375.
- Flaherty KR, Travis WD, Colby TV, Toews GB, Kazerooni EA, Gross BH, Jain A, Strawderman RL, Flint A, Lynch JP, Martinez FJ (2001). Histopathologic variability in usual and nonspecific interstitial pneumonias. *Am J Respir Crit Care Med* 164:1722-1727.
- Franzen P, ten DP, Ichijo H, Yamashita H, Schulz P, Heldin CH, Miyazono K (1993). Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. *Cell* 75:681-692.
- Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR (1996). Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 107:404-411.
- Fujimoto H, Gabazza EC, Hataji O, Yuda H, essandro-Gabazza CN, Nakano M, Franco OE, Hayashi T, Suzuki K, Adachi Y, Taguchi O (2003). Thrombin-activatable

- fibrinolysis inhibitor and protein C inhibitor in interstitial lung disease. *Am J Respir Crit Care Med* 167:1687-1694.
- Furie B, Furie BC (1988). The molecular basis of blood coagulation. *Cell* 53:505-518.
- Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Majno G (1972). Granulation tissue as a contractile organ. A study of structure and function. *J Exp Med* 135:719-734.
- Gajdusek C, Carbon S, Ross R, Nawroth P, Stern D (1986). Activation of coagulation releases endothelial cell mitogens. *J Cell Biol* 103:419-428.
- Gasic GP, Arenas CP, Gasic TB, Gasic GJ (1992). Coagulation factors X, Xa, and protein S as potent mitogens of cultured aortic smooth muscle cells. *Proc Natl Acad Sci U S A* 89:2317-2320.
- Gauldie J (2002). Pro: Inflammatory mechanisms are a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 165:1205-1206.
- Gharaee-Kermani M, Denholm EM, Phan SH (1996). Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors. *J Biol Chem* 271:17779-17784.
- Gharaee-Kermani M, McCullumsmith RE, Charo IF, Kunkel SL, Phan SH (2003). CC-chemokine receptor 2 required for bleomycin-induced pulmonary fibrosis. *Cytokine* 24:266-276.
- Gillery P, Fertin C, Nicolas JF, Chastang F, Kalis B, Banchereau J, Maquart FX (1992). Interleukin-4 stimulates collagen gene expression in human fibroblast monolayer cultures. Potential role in fibrosis. *FEBS Lett* 302:231-234.
- Giri SN, Hyde DM, Hollinger MA (1993). Effect of antibody to transforming growth factor beta on bleomycin induced accumulation of lung collagen in mice. *Thorax* 48:959-966.
- Goffin JM, Pittet P, Csucs G, Lussi JW, Meister JJ, Hinz B (2006). Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J Cell Biol* 172:259-268.
- Goldstein RH, Poliks CF, Pilch PF, Smith BD, Fine A (1989). Stimulation of collagen formation by insulin and insulin-like growth factor I in cultures of human lung fibroblasts. *Endocrinology* 124:964-970.
- Gomperts BN, Strieter RM (2007). Fibrocytes in lung disease. *J Leukoc Biol*.
- Grainger DJ, Wakefield L, Bethell HW, Farndale RW, Metcalfe JC (1995). Release and activation of platelet latent TGF-beta in blood clots during dissolution with plasmin. *Nat Med* 1:932-937.
- Greenberg D, Miao CH, Ho WT, Chung DW, Davie EW (1995). Liver-specific expression of the human factor VII gene. *Proc Natl Acad Sci U S A* 92:12347-12351.
- Gribbin J, Hubbard RB, Le J, I, Smith CJ, West J, Tata LJ (2006). Incidence and mortality of idiopathic pulmonary fibrosis and sarcoidosis in the UK. *Thorax* 61:980-985.
- Griffin CT, Srinivasan Y, Zheng YW, Huang W, Coughlin SR (2001). A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science* 293:1666-1670.

- Grotendorst GR, Duncan MR (2005). Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB J* 19:729-738.
- Gui T, Rehemian A, Funkhouser WK, Bellinger DA, Hagaman JR, Stafford DW, Monahan PE, Ni H (2007). In vivo response to vascular injury in the absence of factor IX: Examination in factor IX knockout mice. *Thromb Res*.
- Gunther A, Lubke N, Ermert M, Schermuly RT, Weissmann N, Breithecker A, Markart P, Ruppert C, Quanz K, Ermert L, Grimminger F, Seeger W (2003). Prevention of bleomycin-induced lung fibrosis by aerosolization of heparin or urokinase in rabbits. *Am J Respir Crit Care Med* 168:1358-1365.
- Gunther A, Mosavi P, Ruppert C, Heinemann S, Temmesfeld B, Velcovsky HG, Morr H, Grimminger F, Walmrath D, Seeger W (2000). Enhanced tissue factor pathway activity and fibrin turnover in the alveolar compartment of patients with interstitial lung disease. *Thromb Haemost* 83:853-860.
- Gurujeyalakshmi G, Giri SN (1995). Molecular mechanisms of antifibrotic effect of interferon gamma in bleomycin-mouse model of lung fibrosis: downregulation of TGF-beta and procollagen I and III gene expression. *Exp Lung Res* 21:791-808.
- Harrison NK, Argent AC, McAnulty RJ, Black CM, Corrin B, Laurent GJ (1991). Collagen synthesis and degradation by systemic sclerosis lung fibroblasts. Responses to transforming growth factor-beta. *Chest* 99:71S-72S.
- Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH (2004). Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 113:243-252.
- Haston CK, Tomko TG, Godin N, Kerckhoff L, Hallett MT (2005). Murine candidate bleomycin induced pulmonary fibrosis susceptibility genes identified by gene expression and sequence analysis of linkage regions. *J Med Genet* 42:464-473.
- Hattori N, Degen JL, Sisson TH, Liu H, Moore BB, Pandrangi RG, Simon RH, Drew AF (2000). Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice. *J Clin Invest* 106:1341-1350.
- Hein L, Ishii K, Coughlin SR, Kobilka BK (1994). Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. *J Biol Chem* 269:27719-27726.
- Heldin CH, Miyazono K, ten DP (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390:465-471.
- Herbert J, Bono F, Herault J, Avril C, Dol F, Mares A, Schaeffer P (1998). Effector protease receptor 1 mediates the mitogenic activity of factor Xa for vascular smooth muscle cells in vitro and in vivo. *J Clin Invest* 101:993-1000.
- Hernandez Rodriguez NA, Cambrey AD, Harrison NK, Chambers RC, Gray AJ, Southcott AM, duBois RM, Black CM, Scully MF, McAnulty RJ (1995). Role of thrombin in pulmonary fibrosis. *Lancet* 346:1071-1073.
- Hetzel M, Bachem M, Anders D, Trischler G, Faehling M (2005). Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts. *Lung* 183:225-237.
- Hinz B (2007). Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 127:526-537.

- Hinz B (2006). Masters and servants of the force: the role of matrix adhesions in myofibroblast force perception and transmission. *Eur J Cell Biol* 85:175-181.
- Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C (2001). Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell* 12:2730-2741.
- Hinz B, Gabbiani G, Chaponnier C (2002). The NH2-terminal peptide of alpha-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo. *J Cell Biol* 157:657-663.
- Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G (2007). The myofibroblast: one function, multiple origins. *Am J Pathol* 170:1807-1816.
- Hinz B, Pittet P, Smith-Clerc J, Chaponnier C, Meister JJ (2004). Myofibroblast development is characterized by specific cell-cell adherens junctions. *Mol Biol Cell* 15:4310-4320.
- Hirano F, Kobayashi A, Hirano Y, Nomura Y, Fukawa E, Makino I (2002). Thrombin-induced expression of RANTES mRNA through protease activated receptor-1 in human synovial fibroblasts. *Ann Rheum Dis* 61:834-837.
- Ho G, Toomey JR, Broze GJ, Jr., Schwartz AL (1996). Receptor-mediated endocytosis of coagulation factor Xa requires cell surface-bound tissue factor pathway inhibitor. *J Biol Chem* 271:9497-9502.
- Hollenberg MD, Compton SJ (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol Rev* 54:203-217.
- Hollenberg MD, Saifeddine M, Al Ani B, Kawabata A (1997). Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. *Can J Physiol Pharmacol* 75:832-841.
- Horvat R, Palade GE (1995). The functional thrombin receptor is associated with the plasmalemma and a large endosomal network in cultured human umbilical vein endothelial cells. *J Cell Sci* 108 ( Pt 3):1155-1164.
- Houliston RA, Keogh RJ, Sugden D, Dudhia J, Carter TD, Wheeler-Jones CP (2002). Protease-activated receptors upregulate cyclooxygenase-2 expression in human endothelial cells. *Thromb Haemost* 88:321-328.
- Howell DC, Goldsack NR, Marshall RP, McNulty RJ, Starke R, Purdy G, Laurent GJ, Chambers RC (2001). Direct Thrombin Inhibition Reduces Lung Collagen, Accumulation, and Connective Tissue Growth Factor mRNA Levels in Bleomycin-Induced Pulmonary Fibrosis. *Am J Pathol* 159:1383-1395.
- Howell DC, Johns RH, Lasky JA, Shan B, Scotton CJ, Laurent GJ, Chambers RC (2005). Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. *Am J Pathol* 166:1353-1365.
- Howell DC, Laurent GJ, Chambers RC (2002). Role of thrombin and its major cellular receptor, protease-activated receptor-1, in pulmonary fibrosis. *Biochem Soc Trans* 30:211-216.
- Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass LF (1993). Internalization and recycling of activated thrombin receptors. *J Biol Chem* 268:13756-13763.
- Hu B, Wu Z, Phan SH (2003). Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *Am J Respir Cell Mol Biol* 29:397-404.



- Huang L, Ogushi F, Tani K, Ogawa H, Kawano T, Endo T, Izumi K, Sono N, Ueno J, Nishitani H, Sone S (2001). Thrombin promotes fibroblast proliferation during the early stages of experimental radiation pneumonitis. *Radiat Res* 156:45-52.
- Huang X, Wu J, Zhu W, Pytela R, Sheppard D (1998). Expression of the human integrin beta6 subunit in alveolar type II cells and bronchiolar epithelial cells reverses lung inflammation in beta6 knockout mice. *Am J Respir Cell Mol Biol* 19:636-642.
- Huang ZF, Higuchi D, Lasky N, Broze GJ, Jr. (1997). Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. *Blood* 90:944-951.
- Hubbard R, Venn A, Smith C, Cooper M, Johnston I, Britton J (1998). Exposure to commonly prescribed drugs and the etiology of cryptogenic fibrosing alveolitis: a case-control study. *Am J Respir Crit Care Med* 157:743-747.
- Hugo C (2003). The thrombospondin 1-TGF-beta axis in fibrotic renal disease. *Nephrol Dial Transplant* 18:1241-1245.
- Hugo C, Pichler R, Meek R, Gordon K, Kyriakides T, Floege J, Bornstein P, Couser WG, Johnson RJ (1995). Thrombospondin 1 is expressed by proliferating mesangial cells and is up-regulated by PDGF and bFGF in vivo. *Kidney Int* 48:1846-1856.
- Hung DT, Vu TH, Nelken NA, Coughlin SR (1992a). Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. *J Cell Biol* 116:827-832.
- Hung DT, Wong YH, Vu TK, Coughlin SR (1992b). The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *J Biol Chem* 267:20831-20834.
- Hung HL, High KA (1996). Liver-enriched transcription factor HNF-4 and ubiquitous factor NF-Y are critical for expression of blood coagulation factor X. *J Biol Chem* 271:2323-2331.
- Hunninghake GW, Hemken C, Brady M, Monick M (1986). Immune interferon is a growth factor for human lung fibroblasts. *Am Rev Respir Dis* 134:1025-1028.
- Hyytiainen M, Penttinen C, Keski-Oja J (2004). Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci* 41:233-264.
- Idell S (2003). Coagulation, fibrinolysis, and fibrin deposition in acute lung injury. *Crit Care Med* 31:S213-S220.
- Idell S, Gonzalez K, Bradford H (1987). Procoagulant activity in bronchoalveolar lavage in the adult respiratory distress syndrome. Contribution of tissue factor associated with factor VII. p. 1466-1474.
- Idell S, James KK, Levin EG, Schwartz BS, Manchanda N, Maunder RJ, Martin TR, McLarty J, Fair DS (1989). Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. *J Clin Invest* 84 AB:695-705.
- Idell S, Kumar A, Zwieb C, Holiday D, Koenig KB, Johnson AR (1994). Effects of TGF-beta and TNF-alpha on procoagulant and fibrinolytic pathways of human tracheal epithelial cells. *Am J Physiol* 267:L693-L703.

- Igarashi A, Okochi H, Bradham DM, Grotendorst GR (1993). Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell* 4:637-645.
- Ignatz RA, Endo T, Massague J (1987). Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. *J Biol Chem* 262:6443-6446.
- Ikeda T, Hirose N, Koto H, Hirano H, Shigematsu N (1989). [Fibrin deposition and fibrinolysis in the pathogenesis of pulmonary fibrosis]. *Nihon Kyobu Shikkan Gakkai Zasshi* 27:448-451.
- Imamura T, Potempa J, Tanase S, Travis J (1997). Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from *Porphyromonas gingivalis*. *J Biol Chem* 272:16062-16067.
- Imokawa S, Sato A, Hayakawa H, Kotani M, Urano T, Takada A (1997). Tissue factor expression and fibrin deposition in the lungs of patients with idiopathic pulmonary fibrosis and systemic sclerosis. *Am J Respir Crit Care Med* 156:631-636.
- Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS (2002a). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 62:65-74.
- Inman GJ, Nicolas FJ, Hill CS (2002b). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Mol Cell* 10:283-294.
- Ishii K, Hein L, Kobilka B, Coughlin SR (1993). Kinetics of thrombin receptor cleavage on intact cells. Relation to signaling. *J Biol Chem* 268:9780-9786.
- Iwai K, Mori T, Yamada N, Yamaguchi M, Hosoda Y (1994). Idiopathic pulmonary fibrosis. Epidemiologic approaches to occupational exposure. *Am J Respir Crit Care Med* 150:670-675.
- Jenkins RG, Herrick SE, Meng QH, Kinnon C, Laurent GJ, McAnulty RJ, Hart SL (2000). An integrin-targeted non-viral vector for pulmonary gene therapy. *Gene Ther* 7:393-400.
- Jenkins RG, Su X, Su G, Scotton CJ, Camerer E, Laurent GJ, Davis GE, Chambers RC, Matthay MA, Sheppard D (2006). Ligation of protease-activated receptor 1 enhances alpha(v)beta6 integrin-dependent TGF-beta activation and promotes acute lung injury. *J Clin Invest* 116:1606-1614.
- Jiang X, Bailly MA, Panetti TS, Cappello M, Konigsberg WH, Bromberg ME (2004). Formation of tissue factor-factor VIIa-factor Xa complex promotes cellular signaling and migration of human breast cancer cells. *J Thromb Haemost* 2:93-101.
- Johnson K, Choi Y, DeGroot E, Samuels I, Creasey A, Aarden L (1998). Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation. *J Immunol* 160:5130-5135.
- Johnston I, Britton J, Kinnear W, Logan R (1990). Rising mortality from cryptogenic fibrosing alveolitis. *BMJ* 301:1017-1021.
- Jones A, Geczy CL (1990). Thrombin and factor Xa enhance the production of interleukin-1. *Immunology* 71:236-241.

- Kahn ML, Hammes SR, Botka C, Coughlin SR (1998). Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *J Biol Chem* 273:23290-23296.
- Kajdacsy-Balla A, Geynisman JM, Macias V, Setty S, Nanaji NM, Berman JJ, Dobbin K, Melamed J, Kong X, Bosland M, Orenstein J, Bayerl J, Becich MJ, Dhir R, Datta MW (2007). Practical aspects of planning, building, and interpreting tissue microarrays: The Cooperative Prostate Cancer Tissue Resource experience. *J Mol Histol* 38:113-121.
- Kaminski N, Allard JD, Pittet JF, Zuo F, Griffiths MJ, Morris D, Huang X, Sheppard D, Heller RA (2000). Global analysis of gene expression in pulmonary fibrosis reveals distinct programs regulating lung inflammation and fibrosis. *Proc Natl Acad Sci U S A* 97:1778-1783.
- Kapanci Y, Desmouliere A, Pache JC, Redard M, Gabbiani G (1995). Cytoskeletal protein modulation in pulmonary alveolar myofibroblasts during idiopathic pulmonary fibrosis. Possible role of transforming growth factor beta and tumor necrosis factor alpha. *Am J Respir Crit Care Med* 152:2163-2169.
- Kapoun AM, Gaspar NJ, Wang Y, Damm D, Liu YW, O'young G, Quon D, Lam A, Munson K, Tran TT, Ma JY, Murphy A, Dugar S, Chakravarty S, Protter AA, Wen FQ, Liu X, Rennard SI, Higgins LS (2006). Transforming growth factor-beta receptor type 1 (TGFbetaRI) kinase activity but not p38 activation is required for TGFbetaRI-induced myofibroblast differentiation and profibrotic gene expression. *Mol Pharmacol* 70:518-531.
- Karnicki K, McBane RD, Miller RS, Leadley RJ, Jr., Morser J, Owen WG, Chesebro JH (2004). Inhibition and reversal of platelet-rich arterial thrombus in vivo: direct vs. indirect factor Xa inhibition. *J Thromb Haemost* 2:2162-2169.
- Kasper M, Haroske G (1996). Alterations in the alveolar epithelium after injury leading to pulmonary fibrosis. *Histol Histopathol* 11:463-483.
- Keane MP, Strieter RM, Lynch JP, III, Belperio JA (2006). Inflammation and angiogenesis in fibrotic lung disease. *Semin Respir Crit Care Med* 27:589-599.
- Keane MP, Wilke CA, Burdick MD, Morris SB, Glass MC, Strieter RM (1999). CXC chemokines regulate angiogenic activity in acute lung injury. *Chest* 116:93S-94S.
- Keerthisingam CB, Jenkins RG, Harrison NK, Hernandez-Rodriguez NA, Booth H, Laurent GJ, Hart SL, Foster ML, McAnulty RJ (2001). Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor-beta in human fibrotic lung fibroblasts and promotes bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* 158:1411-1422.
- Khalil N, Bereznay O, Sporn M, Greenberg AH (1989). Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *J Exp Med* 170:727-737.
- Khalil N, O'Connor RN, Flanders KC, Unruh H (1996). TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am J Respir Cell Mol Biol* 14:131-138.
- Khalil N, O'Connor RN, Unruh HW, Warren PW, Flanders KC, Kemp A, Bereznay OH, Greenberg AH (1991). Increased production and immunohistochemical localization of transforming growth factor-beta in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 5:155-162.

- Kijiyama N, Ueno H, Sugimoto I, Sasaguri Y, Yatera K, Kido M, Gabazza EC, Suzuki K, Hashimoto E, Takeya H (2006). Intratracheal gene transfer of tissue factor pathway inhibitor attenuates pulmonary fibrosis. *Biochem Biophys Res Commun* 339:1113-1119.
- Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA (2006). Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci U S A* 103:13180-13185.
- King TE, Jr., Schwarz MI, Brown K, Tooze JA, Colby TV, Waldron JA, Jr., Flint A, Thurlbeck W, Cherniack RM (2001). Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. *Am J Respir Crit Care Med* 164:1025-1032.
- Kinnula VL, Fattman CL, Tan RJ, Oury TD (2005). Oxidative stress in pulmonary fibrosis: a possible role for redox modulatory therapy. *Am J Respir Crit Care Med* 172:417-422.
- Kirk JM, Heard BE, Kerr I, Turner-Warwick M, Laurent GJ (1984). Quantitation of types I and III collagen in biopsy lung samples from patients with cryptogenic fibrosing alveolitis. *Coll Relat Res* 4:169-182.
- Klages B, Brandt U, Simon MI, Schultz G, Offermanns S (1999). Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* 144:745-754.
- Koizume S, Jin MS, Miyagi E, Hirahara F, Nakamura Y, Piao JH, Asai A, Yoshida A, Tsuchiya E, Ruf W, Miyagi Y (2006). Activation of Cancer Cell Migration and Invasion by Ectopic Synthesis of Coagulation Factor VII. *Cancer Res* 66:9453-9460.
- Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J (2001). Transient expression of IL-1 $\beta$  induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J Clin Invest* 107:1529-1536.
- Kolch W (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J* 351 Pt 2:289-305.
- Kolodsick JE, Toews GB, Jakubzick C, Hogaboam C, Moore TA, McKenzie A, Wilke CA, Chrisman CJ, Moore BB (2004). Protection from Fluorescein Isothiocyanate-Induced Fibrosis in IL-13-Deficient, but Not IL-4-Deficient, Mice Results from Impaired Collagen Synthesis by Fibroblasts. *J Immunol* 172:4068-4076.
- Kondou H, Mushiake S, Etani Y, Miyoshi Y, Michigami T, Ozono K (2003). A blocking peptide for transforming growth factor- $\beta$ 1 activation prevents hepatic fibrosis in vivo. *J Hepatol* 39:742-748.
- Koo BH, Kim DS (2003). Factor Xa induces mitogenesis of vascular smooth muscle cells via autocrine production of epiregulin. *J Biol Chem* 278:52578-52586.
- Kotani I, Sato A, Hayakawa H, Urano T, Takada Y, Takada A (1995). Increased procoagulant and antifibrinolytic activities in the lungs with idiopathic pulmonary fibrosis. *Thromb Res* 77:493-504.
- Kranzhofer R, Clinton SK, Ishii K, Coughlin SR, Fenton JW, Libby P (1996). Thrombin potently stimulates cytokine production in human vascular smooth muscle cells but not in mononuclear phagocytes. *Circ Res* 79:286-294.
- Kubo H, Nakayama K, Yanai M, Suzuki T, Yamaya M, Watanabe M, Sasaki H (2005). Anticoagulant therapy for idiopathic pulmonary fibrosis. *Chest* 128:1475-1482.

- Kucharewicz I, Kowal K, Buczek W, Bodzenta-Lukaszyk A (2003). The plasmin system in airway remodeling. *Thromb Res* 112:1-7.
- Kuhn C, III, Boldt J, King TE, Jr., Crouch E, Vartio T, McDonald JA (1989). An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am Rev Respir Dis* 140:1693-1703.
- Kuhn C, McDonald JA (1991). The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am J Pathol* 138:1257-1265.
- Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* 90:770-774.
- Lakatos HF, Burgess HA, Thatcher TH, Redonnet MR, Hernady E, Williams JP, Sime PJ (2006). Oropharyngeal aspiration of a silica suspension produces a superior model of silicosis in the mouse when compared to intratracheal instillation. *Exp Lung Res* 32:181-199.
- Laurent G, Shapiro S (2007). *Encyclopedia of Respiratory Medicine*. Academic Press.
- Laurent GJ, McAnulty RJ (1983). Protein metabolism during bleomycin-induced pulmonary fibrosis in rabbits. In vivo evidence for collagen accumulation because of increased synthesis and decreased degradation of the newly synthesized collagen. *Am Rev Respir Dis* 128:82-88.
- Laurent GJ, McAnulty RJ, Corrin B, Cockerill P (1981). Biochemical and histological changes in pulmonary fibrosis induced in rabbits with intratracheal bleomycin. *Eur J Clin Invest* 11:441-448.
- Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO (1998). Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest* 101:982-992.
- Lawson WE, Grant SW, Ambrosini V, Womble KE, Dawson EP, Lane KB, Markin C, Renzoni E, Lympay P, Thomas AQ, Roldan J, Scott TA, Blackwell TS, Phillips JA, III, Loyd JE, du Bois RM (2004). Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF. *Thorax* 59:977-980.
- Laxer U, Lossos IS, Gillis S, Or R, Christensen TG, Goldstein RH, Breuer R (1999). The effect of enoxaparin on bleomycin-induced lung injury in mice. *Exp Lung Res* 25:531-541.
- Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten DP (2004). Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* 23:4018-4028.
- Lemay AM, Haston CK (2005). Bleomycin-induced pulmonary fibrosis susceptibility genes in AcB/BcA recombinant congenic mice. *Physiol Genomics* 23:54-61.
- Lenz AG, Costabel U, Maier KL (1996). Oxidized BAL fluid proteins in patients with interstitial lung diseases. *Eur Respir J* 9:307-312.
- Lenz AG, Hinze-Heyn H, Schneider A, Behr J, Haussinger K, Heindi S, Stanzel F, Maier KL (2004). Influence of inflammatory mechanisms on the redox balance in interstitial lung diseases. *Respir Med* 98:737-745.

- Lenzerini L, Benatti U, Morelli A, Pontremoli S, De FA, Piazza A, Rinaldi A, Filippi G, Siniscalco M (1981). Genetic variation in the quantitative levels of an NADP (H)-binding protein (FX) in human erythrocytes. *Blood* 57:209-217.
- Lepailleur-Enouf D, Valdenaire O, Philippe M, Jandrot-Perrus M, Michel JB (2000). Thrombin induces endothelin expression in arterial smooth muscle cells. *Am J Physiol Heart Circ Physiol* 278:H1606-H1612.
- Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G (1976). A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* 17:62-70.
- Light DR, Guilford WJ (2001). Discovery of the factor Xa inhibitor, ZK 807834 (CI-1031). *Curr Top Med Chem* 1:121-136.
- Liu X, Piela-Smith TH (2000). Fibrin(ogen)-induced expression of ICAM-1 and chemokines in human synovial fibroblasts. *J Immunol* 165:5255-5261.
- Ludwicka A, Ohba T, Trojanowska M, Yamakage A, Strange C, Smith EA, LeRoy EC, Sutherland S, Silver RM (1995). Elevated levels of platelet derived growth factor and transforming growth factor-beta 1 in bronchoalveolar lavage fluid from patients with scleroderma. *J Rheumatol* 22:1876-1883.
- Ludwicka-Bradley A, Tourkina E, Suzuki S, Tyson E, Bonner M, Fenton JW, Hoffman S, Silver RM (2000). Thrombin upregulates interleukin-8 in lung fibroblasts via cleavage of proteolytically activated receptor-I and protein kinase C-gamma activation. *Am J Respir Cell Mol Biol* 22:235-243.
- Lwaleed BA, Bass PS (2006). Tissue factor pathway inhibitor: structure, biology and involvement in disease. *J Pathol* 208:327-339.
- Mandal SK, Rao LV, Tran TT, Pendurthi UR (2005). A novel mechanism of plasmin-induced mitogenesis in fibroblasts. *J Thromb Haemost* 3:163-169.
- Mann KG, Brummel K, Butenas S (2003a). What is all that thrombin for? *J Thromb Haemost* 1:1504-1514.
- Mann KG, Butenas S, Brummel K (2003b). The dynamics of thrombin formation. *Arterioscler Thromb Vasc Biol* 23:17-25.
- Manoury B, Nenau S, Leclerc O, Guenon I, Boichot E, Planquois JM, Bertrand CP, Lagente V (2005). The absence of reactive oxygen species production protects mice against bleomycin-induced pulmonary fibrosis. *Respir Res* 6:11.
- Marin V, Farnarier C, Gres S, Kaplanski S, Su MS, Dinarello CA, Kaplanski G (2001). The p38 mitogen-activated protein kinase pathway plays a critical role in thrombin-induced endothelial chemokine production and leukocyte recruitment. *Blood* 98:667-673.
- Marinissen MJ, Servitja JM, Offermanns S, Simon MI, Gutkind JS (2003). Thrombin protease-activated receptor-1 signals through Gq- and G13-initiated MAPK cascades regulating c-Jun expression to induce cell transformation. *J Biol Chem* 278:46814-46825.
- Marsen TA, Simonson MS, Dunn MJ (1995). Thrombin-mediated ET-1 gene regulation involves CaM kinases and calcineurin in human endothelial cells. *J Cardiovasc Pharmacol* 26 Suppl 3:S1-S4.

Martinez FJ, Safrin S, Weycker D, Starko KM, Bradford WZ, King TE, Flaherty KR, Schwartz DA, Noble PW, Raghu G, Brown KK, IPF-Study-Group (2005). The clinical course of patients with idiopathic pulmonary fibrosis. *Annals of internal medicine* 142:963-967.

Massague J, Gomis RR (2006). The logic of TGFbeta signaling. *FEBS Lett* 580:2811-2820.

McAnulty RJ, Campa JS, Cambrey AD, Laurent GJ (1991). The effect of transforming growth factor beta on rates of procollagen synthesis and degradation in vitro. *Biochim Biophys Acta* 1091:231-235.

McAnulty RJ, Laurent GJ (1987). Collagen synthesis and degradation in vivo. Evidence for rapid rates of collagen turnover with extensive degradation of newly synthesized collagen in tissues of the adult rat. *Coll Relat Res* 7:93-104.

McLaughlin JN, Mazzoni MR, Cleator JH, Earls L, Perdigoto AL, Brooks JD, Muldowney JA, III, Vaughan DE, Hamm HE (2005a). Thrombin modulates the expression of a set of genes including thrombospondin-1 in human microvascular endothelial cells. *J Biol Chem* 280:22172-22180.

McLaughlin JN, Shen L, Holinstat M, Brooks JD, Dibenedetto E, Hamm HE (2005b). Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *J Biol Chem* 280:25048-25059.

McLean K, Schirm S, Johns A, Morser J, Light DR (2001). FXa-induced responses in vascular wall cells are PAR-mediated and inhibited by ZK-807834. *Thromb Res* 103:281-297.

Miki H, Mio T, Nagai S, Hoshino Y, Nagao T, Kitaichi M, Izumi T (2000). Fibroblast contractility: usual interstitial pneumonia and nonspecific interstitial pneumonia. *Am J Respir Crit Care Med* 162:2259-2264.

Miller EJ (1985). The structure of fibril-forming collagens. *Ann N Y Acad Sci* 460:1-13.

Mio T, Nagai S, Kitaichi M, Kawatani A, Izumi T (1992). Proliferative characteristics of fibroblast lines derived from open lung biopsy specimens of patients with IPF (UIP). *Chest* 102:832-837.

Mitchell J, Woodcock-Mitchell J, Reynolds S, Low R, Leslie K, Adler K, Gabbiani G, Skalli O (1989). Alpha-smooth muscle actin in parenchymal cells of bleomycin-injured rat lung. *Lab Invest* 60:643-650.

Miyazono K, ten DP, Heldin CH (2000). TGF-beta signaling by Smad proteins. *Adv Immunol* 75:115-157.

Monroe DM, Hoffman M, Roberts HR (2002). Platelets and thrombin generation. *Arterioscler Thromb Vasc Biol* 22:1381-1389.

Monroe DM, Key NS (2007). The tissue factor-factor VIIa complex: procoagulant activity, regulation, and multitasking. *J Thromb Haemost* 5:1097-1105.

Moodley YP, Caterina P, Scaffidi AK, Misso NL, Papadimitriou JM, McAnulty RJ, Laurent GJ, Thompson PJ, Knight DA (2004). Comparison of the morphological and biochemical changes in normal human lung fibroblasts and fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis during FasL-induced apoptosis. *J Pathol* 202:486-495.

Moodley YP, Scaffidi AK, Misso NL, Keerthisingam C, McAnulty RJ, Laurent GJ, Mutsaers SE, Thompson PJ, Knight DA (2003). Fibroblasts isolated from normal lungs and those with idiopathic pulmonary fibrosis differ in interleukin-6/gp130-mediated cell signaling and proliferation. *Am J Pathol* 163:345-354.

Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB (2005). CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. *Am J Pathol* 166:675-684.

Moore BB, Paine R, III, Christensen PJ, Moore TA, Sitterding S, Ngan R, Wilke CA, Kuziel WA, Toews GB (2001). Protection from pulmonary fibrosis in the absence of CCR2 signaling. *J Immunol* 167:4368-4377.

Mori Y, Ishida W, Bhattacharyya S, Li Y, Platanias LC, Varga J (2004). Selective inhibition of activin receptor-like kinase 5 signaling blocks profibrotic transforming growth factor beta responses in skin fibroblasts. *Arthritis Rheum* 50:4008-4021.

Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, Broaddus VC, Nishimura SL (2002). The integrin  $\alpha(v)\beta 8$  mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF- $\beta 1$ . *J Cell Biol* 157:493-507.

Munger JS, Harpel JG, Gleizes PE, Mazziere R, Nunes I, Rifkin DB (1997). Latent transforming growth factor- $\beta$ : structural features and mechanisms of activation. *Kidney Int* 51:1376-1382.

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D (1999). The integrin  $\alpha v \beta 6$  binds and activates latent TGF  $\beta 1$ : a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 96:319-328.

Murphy-Ullrich JE, Hook M (1989). Thrombospondin modulates focal adhesions in endothelial cells. *J Cell Biol* 109:1309-1319.

Murphy-Ullrich JE, Poczatek M (2000). Activation of latent TGF- $\beta$  by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 11:59-69.

Nakao A, Fujii M, Matsumura R, Kumano K, Saito Y, Miyazono K, Iwamoto I (1999). Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *J Clin Invest* 104:5-11.

Naldini A, Carney DH, Pucci A, Pasquali A, Carraro F (2000). Thrombin regulates the expression of proangiogenic cytokines via proteolytic activation of protease-activated receptor-1. *Gen Pharmacol* 35:255-259.

Naldini A, Pucci A, Carney DH, Fanetti G, Carraro F (2002). Thrombin enhancement of interleukin-1 expression in mononuclear cells: involvement of proteinase-activated receptor-1. *Cytokine* 20:191-199.

Naldini A, Sower L, Bocci V, Meyers B, Carney DH (1998). Thrombin receptor expression and responsiveness of human monocytic cells to thrombin is linked to interferon-induced cellular differentiation. *J Cell Physiol* 177:76-84.

Narita M, Rudolph AE, Miletich JP, Schwartz AL (1998). The low-density lipoprotein receptor-related protein (LRP) mediates clearance of coagulation factor Xa in vivo. *Blood* 91:555-560.



ndrade-Gordon P, Derian CK, Maryanoff BE, Zhang HC, Addo MF, Cheung W, Damiano BP, D'Andrea MR, Darrow AL, de GL, Eckardt AJ, Giardino EC, Haertlein BJ, McComsey DF (2001). Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. *J Pharmacol Exp Ther* 298:34-42.

Nicholson AC, Nachman RL, Altieri DC, Summers BD, Ruf W, Edgington TS, Hajjar DP (1996). Effector cell protease receptor-1 is a vascular receptor for coagulation factor Xa. *J Biol Chem* 271:28407-28413.

Noth I, Martinez FJ (2007). Recent advances in idiopathic pulmonary fibrosis. *Chest* 132:637-650.

Oda D, Gown AM, Vande Berg JS, Stern R (1988). The fibroblast-like nature of myofibroblasts. *Exp Mol Pathol* 49:316-329.

Offermanns S, Laugwitz KL, Spicher K, Schultz G (1994). G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proc Natl Acad Sci U S A* 91:504-508.

Offermanns S, Toombs CF, Hu YH, Simon MI (1997). Defective platelet activation in G alpha(q)-deficient mice. *Nature* 389:183-186.

Ohba T, McDonald JK, Silver RM, Strange C, LeRoy EC, Ludwicka A (1994). Scleroderma bronchoalveolar lavage fluid contains thrombin, a mediator of human lung fibroblast proliferation via induction of platelet-derived growth factor alpha-receptor. *Am J Respir Cell Mol Biol* 10:405-412.

Ohta N, Brush M, Jacobs JW (1994). Interaction of antistasin-related peptides with factor Xa: identification of a core inhibitory sequence. *Thromb Haemost* 72:825-830.

Oliver MH, Harrison NK, Bishop JE, Cole PJ, Laurent GJ (1989). A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *J Cell Sci* 92 ( Pt 3):513-518.

Olman MA, Mackman N, Gladson CL, Moser KM, Loskutoff DJ (1995). Changes in procoagulant and fibrinolytic gene expression during bleomycin-induced lung injury in the mouse. *J Clin Invest* 96:1621-1630.

Ossovskaya VS, Bunnett NW (2004). Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* 84:579-621.

Overall CM, Wrana JL, Sodek J (1989). Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoprotease inhibitor expression in human fibroblasts by transforming growth factor-beta. *J Biol Chem* 264:1860-1869.

Pace JM, Corrado M, Missero C, Byers PH (2003). Identification, characterization and expression analysis of a new fibrillar collagen gene, COL27A1. *Matrix Biol* 22:3-14.

Pache JC, Christakos PG, Gannon DE, Mitchell JJ, Low RB, Leslie KO (1998). Myofibroblasts in diffuse alveolar damage of the lung. *Mod Pathol* 11:1064-1070.

Perez RL, Duncan A, Hunter RL, Staton GW, Jr. (1993). Elevated D dimer in the lungs and blood of patients with sarcoidosis. *Chest* 103:1100-1106.

Peyrol S, Cordier JF, Grimaud JA (1990). Intra-alveolar fibrosis of idiopathic bronchiolitis obliterans- organizing pneumonia. Cell-matrix patterns. *Am J Pathol* 137:155-170.

- Phan SH (2002). The myofibroblast in pulmonary fibrosis. *Chest* 122:286S-289S.
- Phan SH, Gharaee-Kermani M, McGarry B, Kunkel SL, Wolber FW (1992). Regulation of rat pulmonary artery endothelial cell transforming growth factor-beta production by IL-1 beta and tumor necrosis factor-alpha. *J Immunol* 149:103-106.
- Phan SH, Zhang K, Zhang HY, Gharaee-Kermani M (1999). The myofibroblast as an inflammatory cell in pulmonary fibrosis. *Curr Top Pathol* 93:173-182.
- Phillips GB, Buckman BO, Davey DD, Eagen KA, Guilford WJ, Hinchman J, Ho E, Koovakkat S, Liang A, Light DR, Mohan R, Ng HP, Post JM, Shaw KJ, Smith D, Subramanyam B, Sullivan ME, Trinh L, Vergona R, Walters J, White K, Whitlow M, Wu S, Xu W, Morrissey MM (1998). Discovery of N-[2-[5-[Amino(imino)methyl]-2-hydroxyphenoxy]-3, 5-difluoro-6-[3-(4, 5-dihydro-1-methyl-1H-imidazol-2-yl)phenoxy]pyridin-4-yl]-N-methylglycine (ZK-807834): a potent, selective, and orally active inhibitor of the blood coagulation enzyme factor Xa. *J Med Chem* 41:3557-3562.
- Phillips PD, Pignolo RJ, Cristofalo VJ (1987). Insulin-like growth factor-I: specific binding to high and low affinity sites and mitogenic action throughout the life span of WI-38 cells. *J Cell Physiol* 133:135-143.
- Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM (2004). Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 114:438-446.
- Plescia J, Altieri DC (1996). Activation of Mac-1 (CD11b/CD18)-bound factor X by released cathepsin G defines an alternative pathway of leucocyte initiation of coagulation. *Biochem J* 319 ( Pt 3):873-879.
- Ploplis VA, Wilberding J, McLennan L, Liang Z, Cornelissen I, Deford ME, Rosen ED, Castellino FJ (2000). A total fibrinogen deficiency is compatible with the development of pulmonary fibrosis in mice. *Am J Pathol* 157:703-708.
- Pohl J, Bruhn HD, Christophers E (1979). Thrombin and fibrin-induced growth of fibroblasts: role in wound repair and thrombus organization. *Klin Wochenschr* 57:273-277.
- Post JM, Sullivan ME, Abendschein D, Ewing J, Hinchman JW, Light DR (2002). Human in vitro pharmacodynamic profile of the selective Factor Xa inhibitor ZK-807834 (CI-1031). *Thromb Res* 105:347-352.
- Postlethwaite AE, Lachman LB, Kang AH (1984). Induction of fibroblast proliferation by interleukin-1 derived from human monocytic leukemia cells. *Arthritis Rheum* 27:995-1001.
- Qi J, Goralnick S, Kreutzer DL (1997). Fibrin regulation of interleukin-8 gene expression in human vascular endothelial cells. *Blood* 90:3595-3602.
- Qian X, Jin L, Lloyd RV (1996). Expression and Regulation of Transforming Growth Factor B1 in Cultured Normal and Neoplastic Rat Pituitary Cells. *Endocr Pathol* 7:77-90.
- Raghow R, Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH (1987). Transforming growth factor-beta increases steady state levels of type I procollagen and fibronectin messenger RNAs posttranscriptionally in cultured human dermal fibroblasts. *J Clin Invest* 79:1285-1288.
- Raghu G (2006). Idiopathic pulmonary fibrosis: treatment options in pursuit of evidence-based approaches. *Eur Respir J* 28:463-465.

- Raghu G, Chen YY, Rusch V, Rabinovitch PS (1988). Differential proliferation of fibroblasts cultured from normal and fibrotic human lungs. *Am Rev Respir Dis* 138:703-708.
- Rahman I, MacNee W (1998). Role of transcription factors in inflammatory lung diseases. *Thorax* 53:601-612.
- Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A (2001). Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. *Am J Respir Cell Mol Biol* 24:591-598.
- Rau JC, Beaulieu LM, Huntington JA, Church FC (2007). Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost* 5 Suppl 1:102-115.
- Reed MJ, Iruela-Arispe L, O'Brien ER, Truong T, LaBell T, Bornstein P, Sage EH (1995). Expression of thrombospondins by endothelial cells. Injury is correlated with TSP-1. *Am J Pathol* 147:1068-1080.
- Renesto P, Si-Tahar M, Moniatte M, Balloy V, Van DA, Pidard D, Chignard M (1997). Specific inhibition of thrombin-induced cell activation by the neutrophil proteinases elastase, cathepsin G, and proteinase 3: evidence for distinct cleavage sites within the aminoterminal domain of the thrombin receptor. *Blood* 89:1944-1953.
- Ribeiro SM, Poczatek M, Schultz-Cherry S, Villain M, Murphy-Ullrich JE (1999). The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta. *J Biol Chem* 274:13586-13593.
- Rice AB, Moomaw CR, Morgan DL, Bonner JC (1999). Specific inhibitors of platelet-derived growth factor or epidermal growth factor receptor tyrosine kinase reduce pulmonary fibrosis in rats. *Am J Pathol* 155:213-221.
- Riewald M, Kravchenko VV, Petrovan RJ, O'Brien PJ, Brass LF, Ulevitch RJ, Ruf W (2001). Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1. *Blood* 97:3109-3116.
- Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W (2002). Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science* 296:1880-1882.
- Riewald M, Ruf W (2001). Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. *Proc Natl Acad Sci U S A* 98:7742-7747.
- Riewald M, Ruf W (2002). Orchestration of coagulation protease signaling by tissue factor. *Trends Cardiovasc Med* 12:149-154.
- Riewald M, Ruf W (2005). Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. *J Biol Chem* 280:19808-19814.
- Rosas IO, Kaminski N (2007). When it comes to genes--IPF or NSIP, familial or sporadic--they're all the same. *Am J Respir Crit Care Med* 175:5-6.
- Rost S, Fregin A, Ivaskavicius V, Conzelmann E, Hortnagel K, Pelz HJ, Lappegard K, Seifried E, Scharrer I, Tuddenham EG, Muller CR, Strom TM, Oldenburg J (2004). Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 427:537-541.

- Rottoli P, Magi B, Perari MG, Liberatori S, Nikiforakis N, Bargagli E, Cianti R, Bini L, Pallini V (2005). Cytokine profile and proteome analysis in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis. *Proteomics* 5:1423-1430.
- Rubtsov YP, Rudensky AY (2007). TGFbeta signalling in control of T-cell-mediated self-reactivity. *Nat Rev Immunol* 7:443-453.
- Ruf W, Dorfleutner A, Riewald M (2003). Specificity of coagulation factor signaling. *J Thromb Haemost* 1:1495-1503.
- Saltzman LE, Moss J, Berg RA, Hom B, Crystal RG (1982). Modulation of collagen production by fibroblasts. Effects of chronic exposure to agonists that increase intracellular cyclic AMP. *Biochem J* 204:25-30.
- Sampson MT, Kakkar AK (2002). Coagulation proteases and human cancer. *Biochem Soc Trans* 30:201-207.
- Sappino AP, Schurch W, Gabbiani G (1990). Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab Invest* 63:144-161.
- Sato E, Koyama S, Masubuchi T, Takamizawa A, Kubo K, Nagai S, Izumi T (1999). Bleomycin stimulates lung epithelial cells to release neutrophil and monocyte chemotactic activities. *Am J Physiol* 276:L941-L950.
- Sato Y, Rifkin DB (1989). Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J Cell Biol* 109:309-315.
- Schultz-Cherry S, Chen H, Mosher DF, Misenheimer TM, Kruttsch HC, Roberts DD, Murphy-Ullrich JE (1995). Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. *J Biol Chem* 270:7304-7310.
- Schultz-Cherry S, Lawler J, Murphy-Ullrich JE (1994). The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. *J Biol Chem* 269:26783-26788.
- Schultz-Cherry S, Murphy-Ullrich JE (1993). Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *J Cell Biol* 122:923-932.
- Scotton C.J., Chambers RC (2007). Molecular targets in pulmonary fibrosis; The myofibroblast in focus.
- Seasholtz TM, Majumdar M, Kaplan DD, Brown JH (1999). Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. *Circ Res* 84:1186-1193.
- Selman M, King TE, Pardo A (2001). Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 134:136-151.
- Selman M, Montano M, Ramos C, Chapela R (1986). Concentration, biosynthesis and degradation of collagen in idiopathic pulmonary fibrosis. *Thorax* 41:355-359.
- Selman M, Pardo A (2006). Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc Am Thorac Soc* 3:364-372.

- Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R, Barrios R, Pardo A (2000). TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? *Am J Physiol Lung Cell Mol Physiol* 279:L562-L574.
- Senden NH, Jeunhomme TM, Heemskerk JW, Wagenvoort R, van't Veer C, Hemker HC, Buurman WA (1998). Factor Xa induces cytokine production and expression of adhesion molecules by human umbilical vein endothelial cells. *J Immunol* 161:4318-4324.
- Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L, Gabbiani G (1998). The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. *J Cell Biol* 142:873-881.
- Serini G, Gabbiani G (1999). Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 250:273-283.
- Seymour ML, Zaidi NF, Hollenberg MD, Macnaughton WK (2003). PAR1-dependent and independent increases in COX-2 and PGE2 in human colonic myofibroblasts stimulated by thrombin. *Am J Physiol Cell Physiol* 284:C1185-C1192.
- Sha X, Yang L, Gentry LE (1991). Identification and analysis of discrete functional domains in the pro region of pre-pro-transforming growth factor beta 1. *J Cell Biol* 114:827-839.
- Shahzeidi S, Jeffery PK, Laurent GJ, McAnulty RJ (1994). Increased type I procollagen mRNA transcripts in the lungs of mice during the development of bleomycin-induced fibrosis. *Eur Respir J* 7:1938-1943.
- Shahzeidi S, Sarnstrand B, Jeffery PK, McAnulty RJ, Laurent GJ (1991). Oral N-acetylcysteine reduces bleomycin-induced collagen deposition in the lungs of mice. *Eur Respir J* 4:845-852.
- Shankar R, de la Motte CA, Poptic EJ, DiCorleto PE (1994). Thrombin receptor-activating peptides differentially stimulate platelet-derived growth factor production, monocytic cell adhesion, and E-selectin expression in human umbilical vein endothelial cells. *J Biol Chem* 269:13936-13941.
- Shapiro MJ, Trejo J, Zeng D, Coughlin SR (1996). Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *J Biol Chem* 271:32874-32880.
- Sheppard D (2006). Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc* 3:413-417.
- Sheppard D (2001a). Integrin-mediated activation of transforming growth factor-beta(1) in pulmonary fibrosis. *Chest* 120:49S-53S.
- Sheppard D (2001b). Pulmonary fibrosis: a cellular overreaction or a failure of communication? *J Clin Invest* 107:1501-1502.
- Shimizu S, Gabazza EC, Hayashi T, Ido M, Adachi Y, Suzuki K (2000). Thrombin stimulates the expression of PDGF in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 279:L503-L510.
- Shinagawa K, Martin JA, Ploplis VA, Castellino FJ (2007). Coagulation factor Xa modulates airway remodeling in a murine model of asthma. *Am J Respir Crit Care Med* 175:136-143.

- Shirato K, Osawa H, Kaizuka M, Nakamura N, Sugawara T, Nakamura M, Tamura M, Yamabe H, Okumura K (2003). Thrombin stimulates production of fibronectin by human proximal tubular epithelial cells via a transforming growth factor-beta-dependent mechanism. *Nephrol Dial Transplant* 18:2248-2254.
- Siflinger-Birnboim A, Johnson A (2003). Protein kinase C modulates pulmonary endothelial permeability: a paradigm for acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 284:L435-L451.
- Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J (1997). Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 100:768-776.
- Siner JM, Jiang G, Cohen ZI, Shan P, Zhang X, Lee CG, Elias JA, Lee PJ (2007). VEGF-induced heme oxygenase-1 confers cytoprotection from lethal hyperoxia in vivo. *FASEB J* 21:1422-1432.
- Singh JP, Adams LD, Bonin PD (1988). Mode of fibroblast growth enhancement by human interleukin-1. *J Cell Biol* 106:813-819.
- Skalli O, Ropraz P, Trzeciak A, Benzouana G, Gillesse D, Gabbiani G (1986). A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 103:2787-2796.
- Smiley ST, King JA, Hancock WW (2001). Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* 167:2887-2894.
- Soslau G, Morgan DA, Jaffe JS, Brodsky I, Wang Y (1997). Cytokine mRNA expression in human platelets and a megakaryocytic cell line and cytokine modulation of platelet function. *Cytokine* 9:405-411.
- Sower LE, Froelich CJ, Carney DH, Fenton JW, Klimpel GR (1995). Thrombin induces IL-6 production in fibroblasts and epithelial cells. Evidence for the involvement of the seven-transmembrane domain (STD) receptor for alpha-thrombin. *J Immunol* 155:895-901.
- Srivastava KD, Magazine HI (1998). Thrombin receptor activation inhibits monocyte spreading by induction of ET(B) receptor-coupled nitric oxide release. *J Immunol* 161:5039-5044.
- Stanton C, Wallin R (1992). Processing and trafficking of clotting factor X in the secretory pathway. Effects of warfarin. *Biochem J* 284 ( Pt 1):25-31.
- Stouffer GA, Runge MS (1998). The role of secondary growth factor production in thrombin-induced proliferation of vascular smooth muscle cells. *Semin Thromb Hemost* 24:145-150.
- Stouffer GA, Sarembock IJ, McNamara CA, Gimple LW, Owens GK (1993). Thrombin-induced mitogenesis of vascular SMC is partially mediated by autocrine production of PDGF-AA. *Am J Physiol* 265:C806-C811.
- Strieter RM (2002). Con: Inflammatory mechanisms are not a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 165:1206-1207.
- Strieter RM (2005). Pathogenesis and natural history of usual interstitial pneumonia: the whole story or the last chapter of a long novel. *Chest* 128:526S-532S.

- Subramanian SV, Polikandriotis JA, Kelm RJ, Jr., David JJ, Orosz CG, Strauch AR (2004). Induction of vascular smooth muscle alpha-actin gene transcription in transforming growth factor beta1-activated myofibroblasts mediated by dynamic interplay between the Pur repressor proteins and Sp1/Smad coactivators. *Mol Biol Cell* 15:4532-4543.
- Sugama Y, Tiruppathi C, offakidevi K, Andersen TT, Fenton JW, Malik AB (1992). Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion. *J Cell Biol* 119:935-944.
- Suganuma H, Sato A, Tamura R, Chida K (1995). Enhanced migration of fibroblasts derived from lungs with fibrotic lesions. *Thorax* 50:984-989.
- Suo Z, Wu M, Ameenuddin S, Anderson HE, Zoloty JE, Citron BA, ndrade-Gordon P, Festoff BW (2002). Participation of protease-activated receptor-1 in thrombin-induced microglial activation. *J Neurochem* 80:655-666.
- Sutherland MR, Raynor CM, Leenknecht H, Wright JF, Pryzdial EL (1997). Coagulation initiated on herpesviruses. *Proc Natl Acad Sci U S A* 94:13510-13514.
- Suzuki T, Moraes TJ, Vachon E, Ginzberg HH, Huang TT, Matthay MA, Hollenberg MD, Marshall J, McCulloch CA, Abreu MT, Chow CW, Downey GP (2005). Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. *Am J Respir Cell Mol Biol* 33:231-247.
- Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA (2000). The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. *Am J Pathol* 157:177-187.
- Tanaka M, Arai H, Liu N, Nogaki F, Nomura K, Kasuno K, Oida E, Kita T, Ono T (2005). Role of coagulation factor Xa and protease-activated receptor 2 in human mesangial cell proliferation. *Kidney Int* 67:2123-2133.
- Tang YW, Johnson JE, Browning PJ, Cruz-Gervis RA, Davis A, Graham BS, Brigham KL, Oates JA, Jr., Loyd JE, Stecenko AA (2003). Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis. *J Clin Microbiol* 41:2633-2640.
- Tani K, Ogushi F, Takahashi H, Kawano T, Endo T, Sone S (1997). Thrombin stimulates platelet-derived growth factor release by alveolar macrophages in rats--significance in bleomycin-induced pulmonary fibrosis. *J Med Invest* 44:59-65.
- Tani KS, Yasuoka F, Ogushi K, Asada K, Fujisawa T, Ozaki T, Sano N, Ogura T (1991). Thrombin enhances lung fibroblast proliferation in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol* 5:34-40.
- ten DP, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K (1993). Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. *Oncogene* 8:2879-2887.
- Thannickal VJ, Aldweib KD, Fanburg BL (1998). Tyrosine phosphorylation regulates H2O2 production in lung fibroblasts stimulated by transforming growth factor beta1. *J Biol Chem* 273:23611-23615.
- Thannickal VJ, Flaherty KR, Martinez FJ, Lynch JP, III (2004). Idiopathic pulmonary fibrosis: emerging concepts on pharmacotherapy. *Expert Opin Pharmacother* 5:1671-1686.

- Todorovic V, Jurukovski V, Chen Y, Fontana L, Dabovic B, Rifkin DB (2005). Latent TGF-beta binding proteins. *Int J Biochem Cell Biol* 37:38-41.
- Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3:349-363.
- Trejo J, Connolly AJ, Coughlin SR (1996). The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. *J Biol Chem* 271:21536-21541.
- Tsao BP, Fair DS, Curtiss LK, Edgington TS (1984). Monocytes can be induced by lipopolysaccharide-triggered T lymphocytes to express functional factor VII/VIIIa protease activity. *J Exp Med* 159:1042-1057.
- Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* 95:779-791.
- Tsunoda S, Yamabe H, Osawa H, Kaizuka M, Shirato K, Okumura K (2001). Cultured rat glomerular epithelial cells show gene expression and production of transforming growth factor-beta: expression is enhanced by thrombin. *Nephrol Dial Transplant* 16:1776-1782.
- Uhl M, Aulwurm S, Wischhusen J, Weiler M, Ma JY, Almirez R, Mangadu R, Liu YW, Platten M, Herrlinger U, Murphy A, Wong DH, Wick W, Higgins LS, Weller M (2004). SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* 64:7954-7961.
- Verleden GM, du Bois RM, Bouros D, Drent M, Millar A, Muller-Quernheim J, Semenzato G, Johnson S, Sourvino G, Olivier D, Pietinalho A, Xaubet A (2001). Genetic predisposition and pathogenetic mechanisms of interstitial lung diseases of unknown origin. *Eur Respir J Suppl* 32:17s-29s.
- Vilcek J, Palombella VJ, Henriksen-DeStefano D, Swenson C, Feinman R, Hirai M, Tsujimoto M (1986). Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. *J Exp Med* 163:632-643.
- Villiger PM, Lotz M (1992). Differential expression of TGF beta isoforms by human articular chondrocytes in response to growth factors. *J Cell Physiol* 151:318-325.
- Vouret-Craviari V, Boquet P, Pouyssegur J, Van Obberghen-Schilling E (1998). Regulation of the actin cytoskeleton by thrombin in human endothelial cells: role of Rho proteins in endothelial barrier function. *Mol Biol Cell* 9:2639-2653.
- Waghray M, Cui Z, Horowitz JC, Subramanian IM, Martinez FJ, Toews GB, Thannickal VJ (2005). Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts. *FASEB J* 19:854-856.
- Wallace WA, Ramage EA, Lamb D, Howie SE (1995). A type 2 (Th2-like) pattern of immune response predominates in the pulmonary interstitium of patients with cryptogenic fibrosing alveolitis (CFA). *Clin Exp Immunol* 101:436-441.
- Wallin R, Turner R (1990). Propeptide recognition by the vitamin K-dependent carboxylase in early processing of prothrombin and factor X. *Biochem J* 272:473-478.
- Walzog B, Weinmann P, Jeblonski F, Scharffetter-Kochanek K, Bommert K, Gaehtgens P (1999). A role for beta(2) integrins (CD11/CD18) in the regulation of cytokine gene



- expression of polymorphonuclear neutrophils during the inflammatory response. *FASEB J* 13:1855-1865.
- Wang Y, Zhou Y, Szabo K, Haft CR, Trejo J (2002). Down-regulation of protease-activated receptor-1 is regulated by sorting nexin 1. *Mol Biol Cell* 13:1965-1976.
- Ward PA, Hunninghake GW (1998). Lung inflammation and fibrosis. *Am J Respir Crit Care Med* 157:S123-S129.
- Ware LB, Camerer E, Welty-Wolf K, Schultz MJ, Matthay MA (2006). Bench to bedside: targeting coagulation and fibrinolysis in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 291:L307-L311.
- Wennerberg K, Rossman KL, Der CJ (2005). The Ras superfamily at a glance. *J Cell Sci* 118:843-846.
- Wilborn J, Crofford LJ, Burdick MD, Kunkel SL, Strieter RM, Peters-Golden M (1995). Cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E2 and to express cyclooxygenase-2. *J Clin Invest* 95:1861-1868.
- Wilcox JN, Noguchi S, Casanova J (2003). Extrahepatic synthesis of factor VII in human atherosclerotic vessels. *Arterioscler Thromb Vasc Biol* 23:136-141.
- Willis BC, duBois RM, Borok Z (2006). Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc Am Thorac Soc* 3:377-382.
- Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z (2005). Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 166:1321-1332.
- Wygrecka M, Markart P, Preissner KT (2007a). Factor XII is a novel mitogenic factor for alveolar epithelial cells in pulmonary fibrosis.
- Wygrecka M, Markart P, Ruppert C, Petri K, Preissner KT, Seeger W, Guenther A (2007b). Cellular origin of pro-coagulant and (anti)-fibrinolytic factors in bleomycin-injured lungs. *Eur Respir J* 29:1105-1114.
- Xu WF, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, Gilbert T, Davie EW, Foster DC (1998). Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci U S A* 95:6642-6646.
- Yamabe H, Osawa H, Inuma H, Kaizuka M, Tamura N, Tsunoda S, Baba Y, Shirato K, Onodera K (1997). Thrombin stimulates production of transforming growth factor- beta by cultured human mesangial cells. *Nephrol Dial Transplant* 12:438-442.
- Yan W, Tiruppathi C, Lum H, Qiao R, Malik AB (1998). Protein kinase C beta regulates heterologous desensitization of thrombin receptor (PAR-1) in endothelial cells. *Am J Physiol* 274:C387-C395.
- Yasui H, Gabazza EC, Taguchi O, Risteli J, Risteli L, Wada H, Yuda H, Kobayashi T, Kobayashi H, Suzuki K, Adachi Y (2000). Decreased protein C activation is associated with abnormal collagen turnover in the intraalveolar space of patients with interstitial lung disease. *Clin Appl Thromb Hemost* 6:202-205.
- Yasui H, Gabazza EC, Tamaki S, Kobayashi T, Hataji O, Yuda H, Shimizu S, Suzuki K, Adachi Y, Taguchi O (2001). Intratracheal administration of activated protein C inhibits

- bleomycin- induced lung fibrosis in the mouse. *Am J Respir Crit Care Med* 163:1660-1668.
- Yehualaeshet T, O'Connor R, Green-Johnson J, Mai S, Silverstein R, Murphy-Ullrich JE, Khalil N (1999). Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. *Am J Pathol* 155:841-851.
- Yoshida M, Sakuma-Mochizuki J, Abe K, Arai T, Mori M, Goya S, Matsuoka H, Hayashi S, Kaneda Y, Kishimoto T (1999). In vivo gene transfer of an extracellular domain of platelet-derived growth factor beta receptor by the HVJ-liposome method ameliorates bleomycin-induced pulmonary fibrosis. *Biochem Biophys Res Commun* 265:503-508.
- Young GD, Murphy-Ullrich JE (2004). Molecular interactions that confer latency to transforming growth factor-beta. *J Biol Chem* 279:38032-38039.
- Yu Q, Stamenkovic I (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 14:163-176.
- Yue TL, Wang XK, Olson B, Feuerstein G (1994). Interleukin-1 beta (IL-1 beta) induces transforming growth factor-beta, (TGF-beta 1) production by rat aortic smooth muscle cells. *Biochem Biophys Res Commun* 204:1186-1192.
- Zhang A, Liu X, Cogan JG, Fuerst MD, Polikandriotis JA, Kelm RJ, Jr., Strauch AR (2005). YB-1 coordinates vascular smooth muscle alpha-actin gene activation by transforming growth factor beta1 and thrombin during differentiation of human pulmonary myofibroblasts. *Mol Biol Cell* 16:4931-4940.
- Zhang G, Kernan KA, Collins SJ, Cai X, Lopez-Guisa JM, Degen JL, Shvil Y, Eddy AA (2007). Plasmin(ogen) promotes renal interstitial fibrosis by promoting epithelial-to-mesenchymal transition: role of plasmin-activated signals. *J Am Soc Nephrol* 18:846-859.
- Zhang HY, Gharaee-Kermani M, Phan SH (1997). Regulation of lung fibroblast alpha-smooth muscle actin expression, contractile phenotype, and apoptosis by IL-1beta. *J Immunol* 158:1392-1399.
- Zhang HY, Gharaee-Kermani M, Zhang K, Karmiol S, Phan SH (1996). Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 148:527-537.
- Zhang HY, Phan SH (1999). Inhibition of myofibroblast apoptosis by transforming growth factor beta(1). *Am J Respir Cell Mol Biol* 21:658-665.
- Zhang K, Flanders KC, Phan SH (1995). Cellular localization of transforming growth factor-beta expression in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 147:352-361.
- Zhang K, Gharaee-Kermani M, Jones ML, Warren JS, Phan SH (1994a). Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. *J Immunol* 153:4733-4741.
- Zhang K, Gharaee-Kermani M, McGarry B, Phan SH (1994b). In situ hybridization analysis of rat lung alpha 1(I) and alpha 2(I) collagen gene expression in pulmonary fibrosis induced by endotracheal bleomycin injection. *Lab Invest* 70:192-202.

---

Zhang K, Rekhter MD, Gordon D, Phan SH (1994c). Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and in situ hybridization study. *Am J Pathol* 145:114-125.

Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, Lollini L, Morris D, Kim Y, DeLustro B, Sheppard D, Pardo A, Selman M, Heller RA (2002). Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. *Proc Natl Acad Sci U S A* 99:6292-6297.

Zywietz A, Gohla A, Schmelz M, Schultz G, Offermanns S (2001). Pleiotropic effects of *Pasteurella multocida* toxin are mediated by Gq-dependent and -independent mechanisms. involvement of Gq but not G11. *J Biol Chem* 276:3840-3845.

## Publications arising from this thesis:

### Full Papers:

1. 'Local extrahepatic upregulation of coagulation factor X: potential novel role in fibrotic lung disease.' **M.A.Krupiczajc**, C.J.Scotton, G.J.Laurent and R.C. Chambers (manuscript in revision with *The Journal of Clinical Investigations*).
2. 'Coagulation Signalling Following Tissue Injury: Focus on the Role of Factor Xa'. **M.A.Krupiczajc**, C.J.Scotton and R.C. Chambers (article accepted for publication: 25-FEB-2008).
3. 'PAR<sub>1</sub> induction of CCL2 in lung epithelial cells'. R.Johns, C.J.Scotton, P.F.Mercer, **M.A.Krupiczajc**, G.J.Laurent and R.C. Chambers (manuscript in revision with *The American Journal of Respiratory Cell and Molecular Biology*).

### Abstracts:

1. 'Local Factor X expression is increased in the injured lung and induces myofibroblast differentiation *in vitro*.'  
**M.A.Krupiczajc**, C.J.Scotton, G.J.Laurent and R.C. Chambers  
American Thoracic Society, May 2006
2. The coagulation cascade in fibrotic lung disease progression: Local expression of factor X is increased in the injured lung.'  
C.J.Scotton, **M.A. Krupiczajc**, R.H. Johns, Y.C.G Lee, M. Koenigshoff, O. Eickelberg, O.Kaminski, G.J. Laurent, R.C.Chambers  
THORAX, VOL 61; Supplement II, pages S104, December 2006
3. 'Activation of PAR<sub>1</sub> by FXa induces fibroblast to myofibroblast differentiation'.  
**M.A.Krupiczajc**, C.J.Scotton, G.J.Laurent and R.C. Chambers,  
Thorax, Vol 60, Supplement II, December 2005
4. 'Factor Xa stimulates lung fibroblast differentiation via activation of PAR<sub>1</sub>'.  
**M.A.Krupiczajc**, C.J.Scotton, G.J.Laurent and R.C. Chambers,  
American Thoracic Society, A60, May 2005

## Academic Awards arising from this thesis:

### 1. International Colloquium in Lung Fibrosis, Germany, 2006

1<sup>st</sup> price Young Investigator Award

### 2. British Lung Foundation/Allen&Hanburys

American Thoracic Society Travel Fellowship 2006.

### 3. Medical Research Society/Academy of Medical Science/Royal College of Physicians

1<sup>st</sup> Poster Prize at the 2006 Meeting for Clinician Scientists in Training.

**4. University College London Graduate School**

*Student Conference Travel Award 2005.*

**5. University College London**

*Rockefeller Studentship 2003.*